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TITLE: Manganese Health Research Program (MHRP)

PRINCIPAL INVESTIGATOR: Michael Aschner, Ph.D.

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203

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14. ABSTRACT

The Administrative Core includes Dr. Michael Aschner of Vanderbilt University Medical Center as the Principal Investigator (PI), and an Administrative Assistant. The Administrative Core is involved in all facets of the MHRP, ensuring proper allocation of funds to the various Research and Support Cores, required financial reporting to the Army, facilitation of the communication between the various CPIs (Core Principal Investigators), timely reporting of the results, communication with all relevant Department of Defense (DoD) personnel, resolving issues related to conflict of interest, and general coordination between the various Cores. A key early objective of the Administrative Core is to allocate, at the direction of the Steering Committee, remaining funds to additional research projects identified. The Administrative Core is advised by the CPIs of the MHRP. Advice includes issues of scientific merit, oversight of ethical issues associated with animal and human subjects, review of potential additional projects, as well as review of funded projects and progress reports.

15. SUBJECT TERMS
Manganese, neurotoxicology, iron deficiency, welding, manganese mining, nutrition

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Introduction and Body

The Steering Committee of the MHRP has met annually (at a minimum) to oversee progress of each of the MHRP projects. Where appropriate, PIs were sent letters for clarification and further requests on the status of their research. As of February 1, 2006, the MHRP contains 15 projects/cores. The original 7 plus 8 projects that were selected in 2006 (see Page 4 for Table of these projects).

The last MHRP meeting took place December 19, 2007 in Washington DC. The meeting was designed to review proposals that were submitted for Phase III of the MHRP, with planned funding commencing February/March, 2008, pending final approval by DoD.

For Phase III solicitation of proposals a letter of intent (LOI) was circulated via various societies and journals (Society of Toxicology, Neurotoxicology, just to name a few) soliciting proposals germane to the MHRP mission by August 2007. Forty letters of intent (see Table, pages 5-9) were received, all of which were reviewed in a telephone conference (October 19, 2007; see Appendix, pages 16-17) attended by the majority of the MHRP Steering Committee (see below for composition). Based on the scientific merit of the letters of intent, the MHRP selected 20 finalists (see Table, pages 10-11) for further consideration requesting a full proposal. Final proposals (NIH format) were invited for late November 25, 2007. The twenty proposals were reviewed December 19 in a special meeting attended by the majority of the MHRP Steering Committee, and 10 finalists (See Table, page 12) were selected for submission to DoD. Presently we are awaiting revisions of the budgets, and once it is complete the PI will submit the full proposal to DoD (planned submission is January, 2008)

The MHRP Steering Committee is chaired by Ms. Anne Tremblay, the Secretary General of the International Manganese Institute (IMnI), and its members include Dr. Barbara Beck of Gradient Corporation, Dr. Tomas Guilarte of the Johns Hopkins University, Dr. Steven Seilkop of SKS Consulting (resigned 2007), Dr. Joan Cranmer of University of Arkansas, Dr. Leonard Levy, MRC Institute for Environment and Health University of Leicester, UK, Dr. Jerry Roper of Afton Chemicals, Dr. Tomas Guilarte of Johns Hopkins University, Sophie Le Pennec of Eramet, France, and Dr. Les Dees of Texas A & M University. The Organizational Chart of the MHRP and the 15 projects that are inherent to the program at present are shown below (see page 13).

The Administrative Assistant, Ms. Alycia Buford-Penn, which was hired originally in 2005 still assumes all the administrative responsibilities for the MHRP. Ms. Buford-Penn joined the MHRP in May 2005. She has trained at Vanderbilt to become versed in all facets of administrative issues germane to this program. She has facilitated communications between the various CPIs and the PI, and has attended to all the administrative issues associated with the MHRP, including reimbursements, reservations for trips, dissemination of pertinent information, etc. Since her tenure with the MHRP started she has had the opportunity to attend numerous computer and university courses related to management and the daily oversight of projects.

MHRP Phase I Projects

1. Administrative Core (Michael Aschner), Vanderbilt University Medical Center
2. Michael Aschner, Vanderbilt University Medical Center
3. Wei Zheng, Purdue University
4. Anne Tremblay, International Manganese Institute, Paris
5. Len Levy, Cranfield University, UK
6. Robert Aitken, Institute of Occupational Medicine, UK 7. David Dorman, North Carolina State University

MHRP Phase II Projects (Funding commenced in February 2006)

1 Neurotoxicity after Pulmonary Exposure to Welding Fumes Containing Manganese -Antonini, James, PI

2 Identification of Novel Biomarkers of Manganese Exposure -Dorman, David, PI

3 A Study of the Nervous System in Welders -Ellingsen, Dag, PI

4 Mechanisms of Manganese-Induced Damage at the Cell and Mitochondrial Level -Gunter, Thomas, PI

5 Role of Manganese in Prion Disease Pathogenesis -Kanthasamy, Anumantha, PI

6 Assessment of Genotoxicity and Carcinogenicity of Inorganic-forms of Manganese -PI Levy, Leonard, PI

7 Role of Toxins and Genetics in Manganese-Induced DA Degeneration -Nass, Richard, PI

8 Molecular Mechanisms Underlying Mn Neurotoxicity -Wu, Jane, PI

- **Forty Two Letter of Intent (LOIs) received for MHRP Phase III**

	Principal Investigator		Submitted institution	Proposal Title
1.	Antonini	James	National Institute for Occupational Safety and Health (NIOSH)	Neurotoxicity of Mn in Welding Fumes in Rats with compromised Liver Function
2	Bowler	Rosemarie	San Francisco State University, San Francisco, CA	Longitudinal Study of Health Effects Over 3 Years in Mn exposed Bridge Welders
3	Bressler	Joseph	UCLA, Los Angeles, California	Indentify Intestinal Transporters for Mn in the Intestine
4	Camps	Manel	Environmental Toxicology, University of California, Santa Cruz, California	Mitochondrial mutagenicity as a Mechanism Contributing to Manganese Neurotoxicity and Carcinogenesis
5	Ceccatelli	Sandra	Karolinska Institute, IMM, Division of Toxicology and Neurotoxicology, Stockholm, Sweden	Assessment of Neurodevelopmental Effects of Manganese
6	Colomina Fosch	M. Teresa	Laboratory of Toxicology and Environmental Health, Rovira I Virgili University, Spain	Teratogen effects of Mn after prenatal exposure
7	Costa	Lucio	Dept. of Environmental and Occupational Health Sciences University of Washington, Seattle, Washington	Effects of Manganese on Glial-Neuronal Interactions
8	Dorman	Dave	Center for Health Research, Triangle Park, North Carolina	Neuroendocrine Effects Associated with Manganese Exposure
9	Erikson	Keith Mallery	Department of Nutrition, The University of North Carolina Greensboro,	The Neurochemical Aspects of Mn Toxicity
10	Felipo	Vicente	Laboratory of Neurobiology, Avda Autopista del Saler, Valencia, Spain	Mechanisms of Manganese damage or repair
11	Filipov	Nick	Center for Environmental Health Sciences Department of Basic Sciences, College of Veterinary Medicine Mississippi State University, MS	Alterations in the Mitochondrial Proteome by Manganese Exposure: in vitro and in vivo Studies
12	Flynn	Mike	University of North Carolina at Chapel Hill	A Pilot Study to Characterize Exposure, Dose, and Effect of Manganese Containing Metal Fumes in the Olfactory Nerve Pathway in Humans
13	Genter	Mary Beth	Department of Environmental Health, University of Cincinnati, Cincinnati, OH	Genetics of Manganese Transport
14	Graziano	Joseph	Environmental Health Sciences, Mailman School of Public Health, Columbia	Water-Borne Manganese Exposure and Motor Function in Young Adults

			University	
15	Guilarte	Tomas	John Hopkins Bloomberg School of Public Health, Baltimore, Maryland	Effects of Manganese in Welding Fumes on Cognitive Function
16	Gunter	Thomas	University of Rochester, Dept. of Biochemistry and Biophysics	Mitochondrial Mn Transport Problems Relevant to Mn Toxicity:
17	Hanneman	William	Dept. of Environmental and Radiological Health Science, college of Veterinary Medicine	The Role of DJ-1 in Mn Induced Neurotoxicity
18	Hirata	Yoko	Dept. of Biomolecular Science, Faculty of Engineering, Gifu University, Japan	The Role of Transport Systems in Selective Toxicity of Manganese on Dopaminergic Cells
19	Iavicoli	Ivo	Catholic University of Sacred Heart, Rome	Neurological Response to Low-Level Long-Term Manganese Exposure in Workers
20	Kanthasamy	Anumantha	Dept. of Biomedical Sciences, Iowa State University, Ames IA	Manganese-Induced Upregulation of Prion Proteins and its Relevance to Prion
21	Klein	Bradley	Dept. of Biomedical Sciences & Pathobiology College of Veterinary Medicine Virginia Tech, Blacksburg, VA	Mitochondrial Substrates for the Differential Toxicity of Manganese in the Nigrostriatal and Mesocortical Dopaminergic Pathways in a Mouse Model of Parkinson's Disease
22	Levy	Leonard	Institute of Environment and Health, Cranfield University,	Funding in Support of the Provision of Research Activity Awareness Services
23	Lucchini	Roberto	Institute of Occupational Health, University of Brescia, Italy	Assessment and Validation of Biological Markers of Manganese Exposure
24	Miller	Gary	Dept. of Environmental and Occupational Health, Emory University, Atlanta, GA	Effects of Manganese Nanoparticles on Subcellular Redox State
25	Milatovic	Dejan	Vanderbilt University Medical Center, Dept. of Pediatric/Toxicology, Nashville, TN	Role of Oxidative Damage and Neurodegeneration in Manganese-Induced Neurotoxicity: Mechanisms and Strategies for Therapeutic Interventions
26	Nagymajtenyi	Laszlo	Dept. of Public Health, University of Szeged, Hungary	Exposure Assessment: Mechanisms determining Mn disposition in the body
27	Niu	Qiao	Shanxi Medical University, P.R. China	Mechanism of transport of Mn into mitochondria

28	Nordberg	Monica	Institute Environmental Medicine, Karolinska Institute Stockholm, Sweden	Manganese in Motor Neuron Disease
29	Olanow	C. Warren	Dept. of Neurology Mount Sinai School of Medicine, New York, NY	A prevalence and risk factor Analysis of Manganism in South African Manganese Smelters
30	Recio	Leslie	Integrated Laboratory Systems, Research triangle Park, NC	Proposal to conduct a GLP-compliant in Vivo Micronucleus Assay According to OECD 474 Guideline and Critical Review of the Genetic Toxicology of Manganese
31	Roth	Jerome	University at Buffalo, Dept. Pharmacology and Toxicology	Parkin Protects Against Manganese Toxicity by Promoting DMT1 Degradation
32	Schneider	Jay	Thomas Jefferson University, Philadelphia, PA	The Manganese Health Research Program Developmental Mn Neurotoxicity: Gene Environment Interactions
33	Smith	Donald	Environmental Toxicology, University of California, Santa Cruz, CA	Manganese Disruption of Iron Metabolism and Regulation: A Fundamental Mechanism of Manganese Neurotoxicity
34	Tanner	Caroline	Clinical Research, Parkinson's Institute, Sunnyvale, CA	Occupational Manganese Exposure and Risk of Parkinsonism: A Case-Control Analysis
35	Tjalkens	Ron	Dept. of Environmental and Radiological Health Sciences College of Veterinary Medicine and Biomedical Sciences Colorado State University, Fort Collins, CO	Glio-vascular Mechanisms in Manganese-Induced Neurologic Dysfunction
36	Tongeren	Martie	Institute of Occupational Medicine, Edinburgh, United Kingdom	Manganese Exposure in and around steel works
37	Wallace	David	Center for Integrative Neuroscience, OSU/CHS, Tulsa, OK	Alterations in the Dopaminergic Transport System Following Low Level Manganese Exposure
38	Wessling-Resnick	Marianne	Dept. of Genetics and Complex Diseases, Harvard School of Public Health, Boston, MA	A Genetic Determinant of Manganese Absorption
39	Westerink	Remco	Institute of Risk Assessment Sciences (IRAS), Utrecht University, The Netherlands	Investigation of the Cellular Mechanisms Underlying Manganese Neurotoxicity in Neuroendocrine Dopaminergic

				Cells
40	Weng	Hsu-Huei	Institute of Occupational Safety and Health, Chang Gung University, Taiwan	The Differences between Parkinsonism due to Manganese Exposure and Idiopathic Parkinson's Disease in Ship Yard Welding Workers
41	Zhang	Li	Dept. of Environmental Health Sciences, Columbia University, New York, NY	Mechanisms underlying neurotoxicity
42	Zheng	Wei	Purdue University, School of Health Sciences, West Lafayette, IN	Brain Magnetic Resonance Imaging (MRI): Relationship to External and Internal Exposure Indices in Manganese-Exposed Smelting Workers

- **MHRP Phase III – 20 Finalist Proposals**

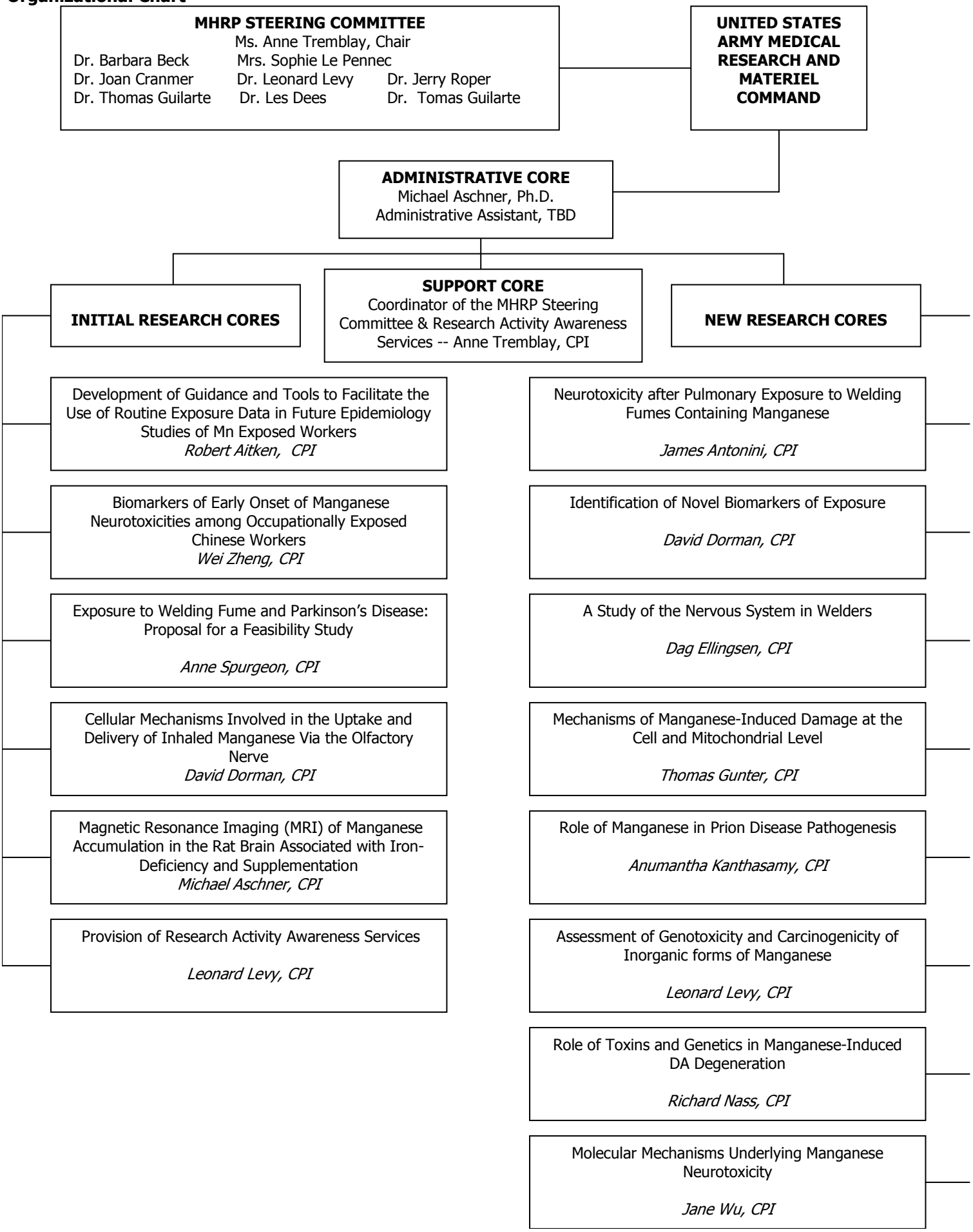
	Principal Investigator		Submitted institution	Proposal Title
1.	Antonini	James	National Institute for Occupational Safety and Health (NIOSH)	Neurotoxicity of Mn in Welding Fumes in Rats with compromised Liver Function
2	Bowler	Rosemarie	San Francisco State University, San Francisco, CA	Longitudinal Study of Health Effects Over 3 Years in Mn exposed Bridge Welders
3	Bressler	Joseph	UCLA, Los Angeles, California	Indentify Intestinal Transporters for Mn in the Intestine
4	Costa	Lucio	Dept. of Environmental and Occupational Health Sciences University of Washington, Seattle, Washington	Effects of Manganese on Glial-Neuronal Interactions
5	Erikson	Keith Mallery	Department of Nutrition, The University of North Carolina Greensboro,	The Neurochemical Aspects of Mn Toxicity
6	Flynn	Mike	University of North Carolina at Chapel Hill	A Pilot Study to Characterize Exposure, Dose, and Effect of Manganese Containing Metal Fumes in the Olfactory Nerve Pathway in Humans
7	Genter	Mary Beth	Department of Environmental Health, University of Cincinnati, Cincinnati, OH	Genetics of Manganese Transport
8	Graziano	Joseph	Environmental Health Sciences, Mailman School of Public Health, Columbia University	Water-Borne Manganese Exposure and Motor Function in Young Adults
9	Guilarte	Tomas	John Hopkins Bloomberg School of Public Health, Baltimore, Maryland	Effects of Manganese in Welding Fumes on Cognitive Function
10	Gunter	Thomas	University of Rochester, Dept. of Biochemistry and Biophysics	Mitochondrial Mn Transport Problems Relevant to Mn Toxicity:
11	Kanthasamy	Anumantha	Dept. of Biomedical Sciences, Iowa State University, Ames IA	Manganese-Induced Upregulation of Prion Proteins and its Relevance to Prion
12	Levy	Leonard	Institute of Environment and Health, Cranfield University,	Funding in Support of the Provision of Research Activity Awareness Services
13	Milatovic	Dejan	Vanderbilt University Medical Center, Dept. of Pediatric/Toxicology, Nashville, TN	Role of Oxidative Damage and Neurodegeneration in Manganese-Induced Neurotoxicity: Mechanisms and Strategies for Therapeutic Interventions

14	Miller	Gary	Dept. of Environmental and Occupational Health, Emory University, Atlanta, GA	Effects of Manganese Nanoparticles on Subcellular Redox State
15	Recio	Leslie	Integrated Laboratory Systems, Research triangle Park, NC	Proposal to conduct a GLP-compliant in Vivo Micronucleus Assay According to OECD 474 Guideline and Critical Review of the Genetic Toxicology of Manganese
16	Roth	Jerome	University at Buffalo, Dept. Pharmacology and Toxicology	Parkin Protects Against Manganese Toxicity by Promoting DMT1 Degradation
17	Schneider	Jay	Thomas Jefferson University, Philadelphia, PA	The Manganese Health Research Program Developmental Mn Neurotoxicity: Gene Environment Interactions
18	Smith	Donald	Environmental Toxicology, University of California, Santa Cruz, CA	Manganese Disruption of Iron Metabolism and Regulation: A Fundamental Mechanism of Manganese Neurotoxicity
19	Tanner	Caroline	Clinical Research, Parkinson's Institute, Sunnyvale, CA	Occupational Manganese Exposure and Risk of Parkinsonism: A Case-Control Analysis
20	Zheng	Wei	Purdue University, School of Health Sciences, West Lafayette, IN	Brain Magnetic Resonance Imaging (MRI): Relationship to External and Internal Exposure Indices in Manganese-Exposed Smelting Workers

- **MHRP Phase III – 10 Proposals Selected for Submission to DoD, January 2008**

	Principal Investigator		Submitted institution	Proposal Title
1	Bowler	Rosemarie	San Francisco State University, San Francisco, CA	Longitudinal Study of Health Effects Over 3 Years in Mn exposed Bridge Welders
2	Costa	Lucio	Dept. of Environmental and Occupational Health Sciences University of Washington, Seattle, Washington	Effects of Manganese on Glial-Neuronal Interactions
3	Graziano	Joseph	Environmental Health Sciences, Mailman School of Public Health, Columbia University	Water-Borne Manganese Exposure and Motor Function in Young Adults
4	Guilarte	Tomas	John Hopkins Bloomberg School of Public Health, Baltimore, Maryland	Effects of Manganese in Welding Fumes on Cognitive Function
5	Gunter	Thomas	University of Rochester, Dept. of Biochemistry and Biophysics	Mitochondrial Mn Transport Problems Relevant to Mn Toxicity:
6	Kanthasamy	Anumantha	Dept. of Biomedical Sciences, Iowa State University, Ames IA	Manganese-Induced Upregulation of Prion Proteins and its Relevance to Prion
7	Levy	Leonard	Institute of Environment and Health, Cranfield University,	Funding in Support of the Provision of Research Activity Awareness Services
8	Milatovic	Dejan	Vanderbilt University Medical Center, Dept. of Pediatric/Toxicology, Nashville, TN	Role of Oxidative Damage and Neurodegeneration in Manganese-Induced Neurotoxicity: Mechanisms and Strategies for Therapeutic Interventions
9	Recio	Leslie	Integrated Laboratory Systems, Research triangle Park, NC	Proposal to conduct a GLP-compliant in Vivo Micronucleus Assay According to OECD 474 Guideline and Critical Review of the Genetic Toxicology of Manganese
10	Zheng	Wei	Purdue University, School of Health Sciences, West Lafayette, IN	Brain Magnetic Resonance Imaging (MRI): Relationship to External and Internal Exposure Indices in Manganese-Exposed Smelting Workers

Organizational Chart



MHRP Symposium at the XXII International Neurotoxicology Conference on Environment and Neurodevelopmental Disorders.

The MHRP also supported a full-day symposium to take place at the XXIII International Neurotoxicity in Development and Aging. The Conference took place September 17-21, 2006 at Little Rock AR. Dr. Joan Cranmer, a member of the MHRP Steering Committee and the Editor of Neurotoxicology has kindly agreed to allocate a full-day for 2 sessions that addressed contemporary issues of Mn. The goal of this symposium was to discuss recent advances in our understanding of molecular mechanisms governing the transport of Mn in the brain, the effects of Mn on specific neuronal systems, the resulting behavioral effects in non-human primates and the discovery of novel biomarkers of Mn exposure in humans. The speakers described a broad spectrum of model systems from the worm *C. elegans*, to mice, non-human primates and humans in a comprehensive approach to increase our understanding of determinants of Mn neurotoxicity. This symposium was multidisciplinary in nature bringing together scientists with expertise in behavioral, molecular, brain imaging and human population studies in the context of mechanistic investigations of Mn neurotoxicity. Participation in this symposium enabled the audience to become acquainted with the latest information and scientific breakthroughs in this fast-paced research area and provided information germane to risk analysis.

During the meeting, the MHRP also held a Steering Committee meeting, which included the majority of its members along with several of the PI, as well as DoD personnel. The minutes of the September 20, 2006 meeting are appended (Please, see Appendix, pages 18-21).

Reportable Outcomes

- Phase I of the MHRP commenced in February 2006 with 8 additional projects, making the total of projects/cores 15 as of this report.
- The Steering Committee met in May, 2007 to discuss progress on each of the MHRP proposals.
- Additional projects were solicited in 2007. Forty two letters of intent were submitted for the August deadline, from those 20 finalists were selected (Telephone conference, October 19 2007) for submission of a full proposal.
- The Steering Committee met on December 19, 2007, and 10 proposals were selected for submission to DoD in January 2008.
- A symposium was held September 17-21, 2006 dedicated to progress of research inherent to the MHRP.

Conclusions

The administrative Core of the MHRP continues to be fully functional. It is successfully involved in all facets of the MHRP, ensuring proper allocation of funds to the various Research and Support Cores, facilitation of the communication between the various CPIs, timely reporting of the results, communication with all relevant Department of Defense (DoD) personnel, resolving issues related to conflict of interest, and general coordination between the various Cores. The Steering Committee has provided input to the MHRP identifying project worthy of funding, and these should commence within the next few weeks. A symposium to gauge the progress of the MHRP took place in September of 2006. Finally, 10 proposals were selected for Phase III of the MHRP with a pending start date of February/March 2008 (pending final approval by DoD).

References

Not applicable

APPENDIX

Pre-proposals Review	
Date: October 16, 2007	
Time: 9:00am est	
Attendees: Aschner, Michael; Roels, Harry; Rousseau, Pierre; Tremblay, Anne; Guilarte, Tomas; Buford-Penn, Alycia; Roper, Jerry; Taylor, Mike; Cranmer, Joan; Beck, Barbara; Levy, Len; Dees, W. Les	
Regrets: Dirk van Niekerk	

Host: Anne Tremblay, Secretary General, International Manganese Institute (IMnI), Paris

Anne Tremblay opened the teleconference meeting welcoming each of the participants

MHRP Phase III Budget-Update: Dr. Michael Aschner

- Funding for MHRP, Phase III, $1.2-1.3 Million available for proposals. Grant will likely be extendable for 1 year, ending February, 2009
- We haven't received payment of $500,000 yet for the original amount allocated.

Conference

- Anne Tremblay suggested that we hold a conference at the end of program to showcase the MHRP's achievements. Everyone agreed
- It was emphasized that the conference will have to take place before February 2009 to allow us to incorporate the costs into the current budget
- Estimated cost for the conference: $200,000
- Anne suggested: use $100,000 from MHRP budget and try to get a grant for the other $100,000

Reviewing Process of Proposals

- Meeting opened up for discussion and review 42 proposals
- Dr. Aschner suggested that each reviewer first nominate proposals for streamlining (no-discussion), followed by a detailed discussion of the best proposals
- All but 20 proposals were streamlined.

- MHRP steering committee members were asked to submit written reviews of the proposals to Anne. These reviews will be added to letters send to PIs which were requested to submit a full proposal.

- Dr. Aschner and Anne Tremblay will draft requests for full proposal letters. The final letters will be sent to Alycia Buford-Penn and then emailed to all PI's on Friday, October 19, 2007 requesting a full proposal. Submission deadline is Sunday, November 25, 2007.

- Guidelines for full proposal submission will adhere to NIH format. The maximum page limit for the proposals (including budgets, references and bios) was set at 8 pages.

- Anne Tremblay will also send 22-thank you letters to Alycia. These will be emailed to PIs that were not selected for consideration of full proposals.

- The MHRP committee will review full proposals December 19, 2007 in Washington, DC (John Hilbert's office).

- Dr. Aschner will submit a Phase III proposal to Department of Defense by end of the year for final consideration and approval.

Conference ended: Total of 5 hours conference call

MHRP The Manganese Health Research Program	
Date: Wednesday, September 20, 2006	**Location:** Double Tree Hotel Little Rock, AR
Time: 5:00pm – 7:00pm	
Attendees: Aschner, Michael; Tremblay, Anne; Hilbert, John; Kanthasamy, Anumantha; Guilarte, Tomas; Gunter, Thomas; Kundakjian, Patricia; Seilkop, Steven; Buford-Penn, Alycia; Roper, Jerry; Antonini, James; Taylor, Michael; Vigneulle, Roy; Hover, Carl; Nass, Richard; Cranmer, Joan	
Regrets: Barbara Beck , Len Levy, Dirk van Niekerk	

5:00p – 5:10p ……..Introductions

Dr. Aschner opened the meeting with introductions of Guest Panel and MHRP members.

5:10 – 5:20 PM (POWERPOINT PRESENTATION)
MHRP - Origin and Industry Perspective
Anne Tremblay, Secretary General, International Manganese Institute (IMnI), Paris

- Anne Tremblay reviewed the origins of the MHRP and described the industry's expectations for the program.
- **What is IMnI?** - It's an association of Mn ore and alloy producers. A few research institutes are also members of the IMnI. Currently sixty-two members, operating in 29 countries are affiliated with the IMnI.
- **IMnI is in the process of defining a new Mission Statement:** IMnI provides vision and guidance to the manganese industry by promoting economic, social and environmental responsibility and sustainability for all stakeholders
- **What This Means:** IMnI helps keep the industry informed, proactive and responsible for Mn associated issues.
- In 2001, IMnI retained services of Kinghorn, Hilbert & Associates (John Hilbert's lobbying firm) aiming to raise $6 million over a 3 yr. period. This led to a lobbying effort, resulting in the establishment of the MHRP via Department of Defense (DoD) funding. To date, MHRP has been a beneficiary of $5.05 million over 3 years (FY04, FY05 and FY06).
- This year IMnI decided to lobby for a 4th year of funding in hopes that DoD would integrate MHRP program into its own budget for FY08.
- IMnI would like to see the partnership between DoD and Vanderbilt University result in future independent requests for funds from "traditional' sources, such as NIH and NSF.
- **Closing remarks**

Special thanks*:* United States Dept. of Defense, Col Brian Lukey & Col. Carl Hover; United States Dept. of Defense; Dr. Michael Aschner, Principal Investigator, Vanderbilt University Medical Center; John Hilbert, Kinghorn, Hilbert & Associates; Board of IMnI.

- For additional information, please refer to website http://www.manganese.org/

5:20 – 5:35 PM (POWERPOINT PRESENTATION) Lobbying Efforts John Hilbert, President, Kinghorn, Hilbert & Associates, LLC, Washington, DC

- Attorney John Hilbert briefed the attendees on the process associated with his firm's lobbying efforts.

Update Funding for MHRP
FY 04, $1.4 M,
FY 05, $2.25 M
FY 06, $1.4 M
TOTAL 5.05 MILLION (Congressional appropriations)

- Congress appropriations - this year (FY 07) a $2.7 Million dollar request was made for the MHRP, with support of multiple Members of Congress.

- In January, request was made to congress by three companies, who are members of IMnI, **1. Afton Chemical 2. Energizer Battery Company 3. Erachem.**

- The next phase of support was envisioned to be a request by Col Lukey or Col Hover of $3Million to be added to their (DoD) budget for FY08.

- Alternative Funding - John Hilbert's firm contacted The National Science Foundation (NSF). NSF requested a letter of support for the MHRP from few members of congress and from the President of Vanderbilt University. Grants from NSF are typically $1 to $3 million range. John Hilbert stated, they will have congressional support for an NSF grant request to support the MHRP.

- **Closing remarks:** John Hilbert stated this was a difficult year on Capitol Hill, and that additional resources of revenues will have to be found in order to continue the MHRP.

5:35 – 5:50 PM. (POWERPOINT PRESENTATION)
Military Relevance of MHRP and Expectations for Deliverables
Col. Carl Hover, USAMRMC
Ft. Detrick, Maryland

- Col. Carl Hover is the director of Military Operational Medicine Research Program (**MOMRP**).

- **PROGRAM SUMMARY:**

MOMRP mission is to provide biomedical solutions to protect soldiers and enhance war fighters capabilities.

- Col Hover discussed funds available to MOMRP, the appropriations processes and the priorities of his office. His statements suggested that the MHRP is better off to obtain funding via congressional appropriations rather than a line item budget within his directed programs.

5:45 – 6:00 PM
MHRP - Scientific Perspective
Michael Aschner, Vanderbilt University Medical Center

Dr. Aschner's perspective of MHRP

- Dr. Aschner stated that the MHRP has and will make a difference in the understanding of Mn-induced disease. Year one of the project started out with seven projects/cores, five of which are research

oriented and two of which are administrative cores. In FY05 and FY06, 8 additional research projects were added, bringing the total to 15. Dr. Aschner described the status of the various projects and alluded to some difficulties in getting a few of those to run smoothly, primarily as a result of delays associated with clearance of Institutional Review Boards (IRBs), approving human studies in China, Russia and the UK. In general all the research projects that utilize animals as experimental models are at various stages of study, while most of the human studies have started after some significant delays. Overall, Dr. Aschner believes that these delays will not affect the time table of the MHRP, and results should be available for dissemination in a timely fashion.

- **Closing remarks:** Dr. Aschner thanked DoD for sponsoring the MHRP Program Conference and to the members of the MHRP Steering Committee.

6:00 – 7:00 PM - Open Discussion - All participants

Roy Vigneulle - offered to assist people to find federal funding dollars.

Dr. Aschner - stated the need to be more creative, selective and persistent to allow for the continuity of the MRHP.

Joan Cranmer - suggested that after investment in the MHRP, it seems reasonable that the DoD would want to maintain this umbrella of scientists intact.

Jerry Roper - suggested a conference in Washington, DC, to showcase results.

7:00PM - MHRP Dinner @ Bosco's, 500 President Clinton Ave.
501-907-1881

Attendees:
Aschner, Michael; Aschner, Judy; Tremblay, Anne; Hilbert, John; Kanthasamy, Anumantha; Guilarte, Tomas; Gunter, Thomas; Koundakjian, Patricia; Seilkop, Steven; Roper, Jerry; Antonini, James; Taylor, Michael; Vigneulle, Roy; Hover, Carl; Nass, Richard; Cranmer, Joan; Mergler, Donna

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AWARD NUMBER: W81XWH-05-1-0239

TITLE: Magnetic Resonance Imaging (MRI) of Manganese Accumulation in the Rat Brain Associated with Iron-Deficiency and Supplementation

PRINCIPAL INVESTIGATOR: Michael Aschner, PhD and Vanessa A. Fitsanakis, PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203

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Fitsanakis, Vanessa, A., PhD

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14. ABSTRACT

Manganese (Mn) and iron are essential metals for normal growth and development that compete for and share the same transporters. Thus, during periods of low dietary iron intake, the transport and deposition of Mn in the brain are increased. Conversely, high-risk populations for Mn intoxication, namely Mn miners and welders, may benefit from iron supplementation, which may lower their central nervous system (CNS) Mn burden. For the first 2 years, we proposed to determine the temporal brain Mn deposition pattern using magnetic resonance imaging (MRI). We have completed the imaging and atomic absorption spectroscopy (AAS) phases. Both iron and Mn content in six discrete brain regions have been determined, along with ascertainment of blood and plasma metal levels. Data analysis is progressing for both the brain images and R1 values from the MRI, and several manuscripts have been submitted, both addressing Mn and Fe modeling in the brain, as well as the relationship between Mn and Fe brain depositions. Studies on the effects of Mn on dopaminergic function, with MR Spectroscopy (MRS) are in progress.

15. SUBJECT TERMS
Manganese, neurotoxicology, iron deficiency, welding, manganese mining, nutrition

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Introduction

Manganese (Mn) is an essential metal for normal growth and development. Recent work demonstrates that Mn and iron (Fe) compete for and share the same transporters. Thus, during periods of low dietary Fe intake, the transport and deposition of Mn in the brain are increased. Conversely, high-risk populations for Mn intoxication, namely Mn miners and welders, may benefit from Fe supplementation, which may lower their central nervous system Mn burden. Given the potential health risks associated with Mn neurotoxicity, we proposed using a non-invasive *in vivo* technique, magnetic resonance imaging (MRI), to monitor the accumulation of brain Mn when dietary levels of Fe are modulated. However, before Fe levels could be experimentally manipulated in animal models, it was first necessary to determine the usefulness of MRI in monitoring brain Mn levels in the absence of changes in dietary Fe.

In this phase of the proposal, we fed adult rats, with or without Mn injections, normal levels of dietary Fe. Temporal brain Mn deposition was assessed with MRI throughout the study. Blood was collected from animals at various time points so that levels of Fe, Mn, transferrin and ferritin could be determined. Specifically, we were interested in correlating blood and brain Mn concentrations with either MR image intensities or rate constants determined during the course of Mn treatment.

The second phase of this study, namely manipulation of dietary Fe levels, is the first attempt to utilize MR for monitoring *both* brain Mn deposition and the role of Fe deficiency or supplementation in this process. Findings derived from these studies will address the potential risk for increased Mn deposition in the brain of Fe-deficient human populations, as well as the potential of Fe supplementation to diminish brain Mn accumulation in populations already identified as vulnerable to Mn intoxication. Significantly, if these studies document a relationship between Fe status and Mn deposition, they will provide a rationale and impetus for future studies on the possibility that high Fe ambient air (such as in welding fumes) may also protect the brain from increased Mn deposition.

Body

STUDY 1 – MRI Modeling

Mn and Fe are both paramagnetic species that can affect magnetic resonance relaxation rates. They also share common transport systems in vivo and thus in experimental models of metal exposure their effects on relaxation rates may interact in a complex fashion. Here we present a novel model to interpret the combined effects of Mn and Fe on MRI relaxation rates. To achieve varying levels of both metals, adult rats were separated into 4 groups; a control group and three groups treated with weekly intravenous injections of 3 mg Mn/kg body for 14 weeks. The three treated groups were fed either a normal diet, Fe deficient or Fe enriched diet. All rats were scanned using MRI at the 14^{th} week to measure regional water relaxation rates. Rat brains were removed at the end of the study (14^{th} week) and dissected into regions for measurement of Mn and Fe by atomic absorption spectroscopy. For the normal diet groups, R_1 was strongly correlated with tissue Mn concentrations. However, the slopes of the linear regression fits varied significantly among different brain regions, and a simple linear model failed to explain the changes in relaxation rate when both Mn and Fe contents changed. We propose a more complex model based on the fact that Mn and Fe may compete in vivo to explain how they may affect MRI signals when the levels of both metals vary.

Comparison of the competition model with the linear models

The fits to the data for the linear and competition models have been compared. Also, the regressions for all brain regions for all four models (competition model, linear model with both Mn and Fe considered, linear model with only Mn considered, linear model with only Fe considered) were assessed. The competition model and the linear model with Mn and Fe fitted together using specific equations provided the best r^2 in all of the brain regions. In some brain regions the fits derived by these two models are significantly improved over the other two linear models in which only one ion is considered. For example, for striatum the correlation coefficients derived by the competition and the linear model with both Mn and Fe considered are 0.87 and 0.85 respectively, while the values for the other two linear model with either Mn or Fe considered are 0.69 and 0.02 with 95% confidence interval of [0.36, 0.86] and [-0.42, 0.45] respectively. Other brain regions like brainstem

and midbrain also provide evidence that the competition and the linear model taking account of both Mn and Fe provide better fitting results than one or both of the other two linear models. There is no significant difference between the correlation coefficients of the competition model and the linear model with both Mn and Fe counted. However, the relaxivities of Fe fitted with the linear model are significantly negative in almost all of the brain regions, which do not have any physical meaning. Thus, considering one metal alone at a time or both acting independently fails either to provide physically interpretable fits or to fit the data very successfully. Only by considering the competitive model are reasonable fits that have physical meanings offered.

A diet with Fe overload is known to increase Fe accumulation in the plasma. The increased brain Mn concentration accompanied by decreased Fe concentration with Fe supplements is surprising. This may be due to the fact that the added Mn that is on board in the FeSMnT group is more readily taken into the brain, offsetting the Fe overload in the diet.

It likely is associated with the inability of the regulatory machinery to accurately reflect the brain's Fe requirements due to transport and storage of Fe in ferritin. Normally, in the presence of exceedingly high levels of Fe, the regulatory pathway perceives the CNS as Fe deficient despite excessive Fe accumulation and Fe uptake into the brain continues. When Fe regulatory protein-1 (IRP1) and IRP2 bind to the Fe regulatory element (IRE) in the 3′-untranslated region of transferrin receptor (TfR) or DMT1 mRNA, the transcript is stabilized, translation proceeds, and the proteins are synthesized. Thus, a high IRP binding activity reflects low body Fe stores and results in up-regulation of DMT1 and TfR. Vice versa, high intracellular Fe concentrations would have an opposite effect. Nonetheless, it is possible that the down-regulation of DMT1 and TfR is associated with up-regulation of transporters that are Mn-specific. Thus even in the presence of high Fe, the uptake of Mn may unabatedly continue. It has also been reported by Chua and Morgan that iron overload and deficiency led to increased brain Mn.

Regional variation of the relaxivities and combined influence of Mn and Fe on MRI signal

The effects of Mn and Fe on MR relaxation rates have been studied before in isolation and without consideration of the potential interaction between the two. Thus, when studies are designed to examine the effect of Mn on relaxivities, no other metal ions are usually considered. Conversely, other studies that focus solely on Fe have failed to take into account the interrelationship of this metal with Mn. As a result, most researchers have used a linear model to explain the influence of paramagnetic ions on the MRI signal. Our results show that when only one paramagnetic ion concentration change occurs, the simple linear model may appear to explain the relationship between ion concentration and relaxation rates. Thus the relaxation rate measured by MRI can be used as an indicator of ion concentration for this case. However, even with this simple linear model, the relaxivities vary among regions, implying that different brain regions should be treated separately rather than taking the whole brain as a single homogeneous region. To our knowledge, our study is the first one to examine the regional variation of the relaxivities.

Conclusions

Although Mn and Fe are commonly studied together in the toxicology field, no MRI study has been found in the literature that reports the combined influence of Mn and Fe on MRI signals. Our study is the first one to investigate this effect. Our results reveal that when more than one paramagnetic ion concentration is changing, the linear model does not describe the effects properly. As a result, a more complicated model must be applied. We propose an interacting model based on the fact that Mn and Fe may compete in vivo and both of them will affect MRI signals. The regional variation effect is still very apparent in the interacting model.

STUDY 2 – Determination of brain Mn under conditions of changes in dietary Fe

Chronic exposure to manganese (Mn) may lead to a movement disorder due to preferential Mn accumulation in the globus pallidus and other basal ganglia nuclei. Iron (Fe) deficiency also results in increased brain Mn levels, as well as dysregulation of other trace metals. The relationship between Mn and Fe transport has been attributed to the fact that both metals can be transported via the same molecular mechanisms. It is not known, however, whether brain Mn distribution patterns due to increased Mn exposure vs. Fe deficiency are the same,

or whether Fe supplementation would reverse or inhibit Mn deposition. To address these questions, we utilized four distinct experimental populations. Three separate groups of male Sprague-Dawley rats on different diets [control diet (MnT), Fe deficient (FeD) or Fe supplemented (FeS)] were given weekly intravenous Mn injections (3 mg Mn/kg body mass) for 14 weeks, while control (CN) rats were fed the control diet and received sterile saline injections. At the conclusion of the study, both blood and brain Mn and Fe levels were determined by atomic absorption spectroscopy (AAS) and magnetic resonance imaging (MRI)(Examples of MRI data are shown in Figure 1). The data indicate that changes in dietary Fe levels (either increased or decreased) result in regionally specific increases in brain Mn levels compared to CN or MnT animals. Furthermore, there was no difference in either Fe or Mn accumulation between FeS or FeD animals. These data suggest that dietary Fe manipulation, whether increased or decreased, may contribute to brain Mn deposition in populations vulnerable to increased Mn exposure.

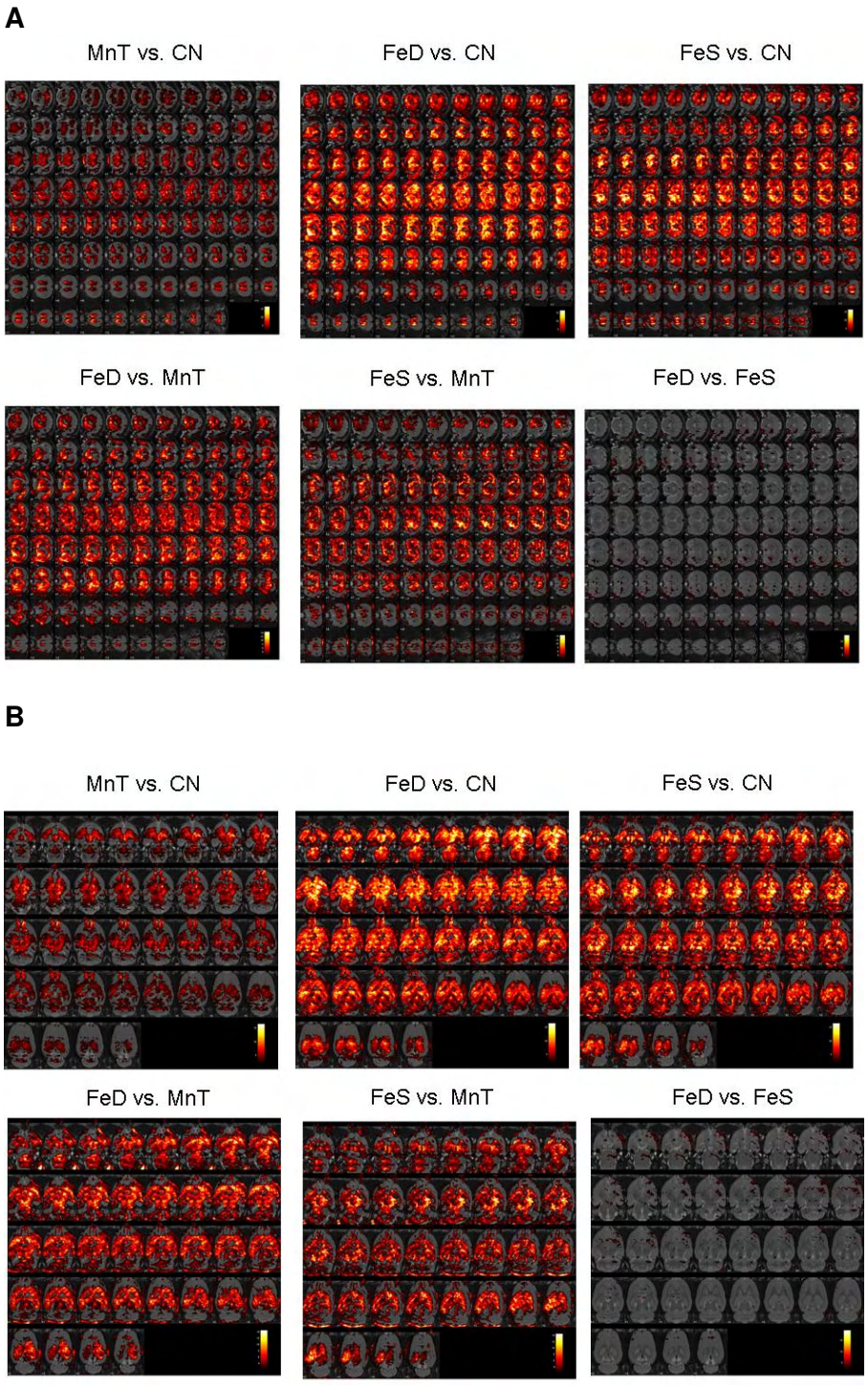


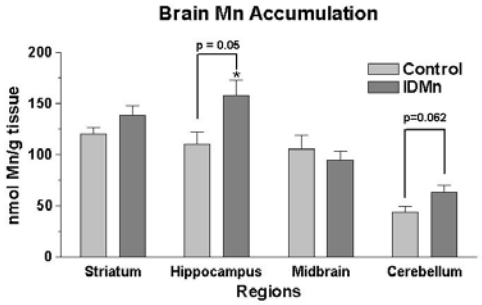
Figure 1: Magnetic Resonance (MR) Images Depicting Brain Mn Accumulation—Animals were imaged at week 14 to determine regional changes in brain Mn deposition. T1 maps were compared across groups and the corresponding statistical maps were overlaid on high resolution rat brain templates. Images are presented in the (a) frontal, and (b)

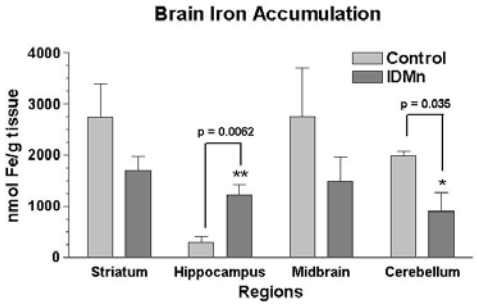
horizontal. White indicates greater statistically significant differences in regional Mn amounts, while red faux coloring indicates the minimal threshold of $p < 0.05$.

Conclusion:

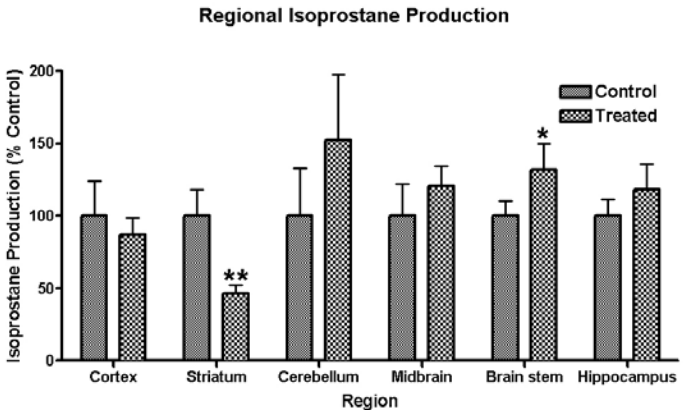
It has been shown above, when Mn and Fe concentrations are both altered in a biological system, that their combined influence on MRI signals is complicated. In such a case, the simple linear model for explaining the relationship between MRI signal and a single changed paramagnetic ion will not be suitable to explain the change in MRI relaxation rates. Regional variations are apparent in both the experimental data and the model. Although some limitations and uncertainty exist in our model such as the presence of several free parameters in the fit, this represents a first attempt to explain the interacting relationship of two paramagnetic ions and their influence on the MRI signals. Our model correctly predicts the nonlinear relationship between relaxation rates and ion contents. This model may be useful for interpreting MR results when more than one paramagnetic species is involved.

STUDY 3 – Effects of Mn and dietary Fe manipulations on behavior and oxidative stress in the rat brain





As pregnant women are more likely to suffer from ID than other groups, we utilized pregnant Sprague-Dawley dams, following birth of viable litters, fed an IDMn diet both during pregnancy and for 42 weeks beyond giving birth. General body mass, clinical blood parameters, brain metal levels and two behavioral tests valid to cognitive function were conducted to assess the effects of this treatment in the IDMn adults compared to CN animals. The treatment protocol was devised in order to produce only mild ID, and the blood data demonstrate that IDMn animals tended to have reduced Hb and Fe, with higher Tf and TIBC, without inducing full-blown anemia (see Figure to the left). Additionally, brain Fe levels were generally decreased in the entire brain, consistent with consumption of a Fe deficient diet. Interestingly, by the last week of treatment, blood parameters appeared to be approaching more normal levels. This likely indicates that homeostatic mechanisms in these dams were returning to a healthy, more CN-like phenotype. Although we were able to detect regional variations in brain IsoP levels, it is difficult to interpret this data. This could be due to the fact that the tissue preparation was not optimized for this analysis. However, when regional brain metal data is compared with IsoP data, it is interesting to note that decreased striatal Fe levels mirrored decreased production of IsoPs, although the same trend was not observed for the cerebellum. Thus it appears that, while increased oxidative stress may be occurring in the striatum, midbrain and brain stem of treated animals, it may not directly correlate to brain metal levels. It is known that dietary changes in Mn and Fe levels not only perturb these metals, but many others as well. It is likely the case, then, that the relationship between Fe and/or Mn and IsoP levels is not as straightforward as originally anticipated.



While it is known that severe ID in humans leads to decreased cognitive functioning in infants and children, we wanted to determine whether similar phenomena are also observed in adult female animals. Consistent with data from younger animals, our adult females also demonstrated impaired cognitive performance on the MWM as compared to CNs. Regional brain metal analysis indicated that both Mn and Fe deposition were significantly altered in

treated rats, particularly in the hippocampus. Conversely, hippocampal levels of IsoPs, used as a marker for oxidative stress, were not statistically significantly different in IDMn dams compared to CN rats (see Figure to the left). Interestingly, there were no statistically significant effects on auditory sensorimotor gating as measured by PPI, which has also been shown to be affected by losses of function in the hippocampus or hippocampal formation, although PPI function appears to be mediated by a complex circuit involving basal forebrain structures, the frontal cortex, as well as areas of the brain stem. Specific to the hippocampus, studies have shown that PPI function appears to be primarily affected by ablation of the ventral hippocampus, but only if the lesion is given during development, whereas MWM performance has been shown to be more susceptible to damage of the dorsal, but not ventral, hippocampus. These behavioral data may indicate that the dorsal hippocampus is more vulnerable to iron deficiency. Thus, cognitive impairments may be more directed at tasks involving complex cognitive associations related to the dorsal hippocampus *versus* a disruption in development of the ventral hippocampus and related structures, which appears to be important in sensorimotor gating. This hypothesis is speculative at this point, and different regions of the hippocampus must be analyzed to determine whether iron deficiency is more disruptive to the dorsal hippocampus *versus* other subregions of this structure.

Regarding MWM performance, there were no significant effects on acquisition of the platform location, but there were significant deficits in IDMn rats on the probe trial as compared to controls. Studies have shown that rats with a brain insult, if given the appropriate training procedures, may not demonstrate deficits on acquisition latency, but rather show poor performance on the probe trial at the end of training. Although this may seem contradictory, several studies have demonstrated dissociation between acquisition latency and probe trial performance in rats tested on the MWM. Whishaw et al. (1995) have distinguished between the ability to reach the platform during acquisition and probe trial performance as "getting there" and "knowing where". In essence, rats can learn a strategy to navigate to the platform during acquisition, such as a motor or landmark strategy, but may not necessarily have a cognitive representation of where the platform is located. Hypothetically, if an animal has memorized a strategy to locate the platform during acquisition and the probe trial is administered, the strategy of memorizing a set of movements to reach the platform location will fail once the animal reaches the former platform site. Conversely, a rat with knowledge of the former platform location on the probe trial can revert to several different strategies, including extra-maze cue associations, path integration, allothetic or kinesthetic cues, as well as other previously successful search strategies. In this experiment, it appears that IDMn rats were able to learn a specific strategy to locate the platform during acquisition, but did not have the ability to integrate these strategies on the probe trial. This suggests that iron-deficiency compounded by high Mn levels may result in an inability to integrate information on behavioral tasks that are more demanding on the cognitive system,

Conclusions

Our data suggest that and IDMn diet is useful in producing mild ID, without over anemia, in dams during and following pregnancy. Additionally, this treatment leads to altered regional brain deposition of both Mn and Fe, which resulted in spatial memory deficits in a behavioral task requiring complex cognitive associations. These data suggest that, while blood Fe parameters in IDMn animals may approach CN levels after almost one year of treatment, lingering cognitive changes may persist in adult females long after pregnancy. More studies need to be completed to demonstrate that reversal of the dietary changes also lead to a reversal of cognitive impairments. However, our data suggest that vulnerable ID populations exposed to high levels of Mn are indeed at risk of potentially dangerous alterations in brain metal levels.

Key Research Accomplishments

- Rats were fed normal diets, with treated animals receiving weekly injections of $MnCl_2$ for 14 weeks, with no significant toxicity.

- Both control and treated groups were scanned prior to start of treatment to obtain baseline MR images. Scanning continued at weeks 1, 3, 5, 7, 9, 11, 13 and 14. This allowed for successful comparison of images between groups as well as within groups at various time points.

- Blood samples were taken at weeks 8 and 14 for determination of both Mn and Fe levels.

- At the conclusion of the study, animals were humanely euthanized and brains were removed and dissected into the following regions: cerebellum, brain stem (pons and medulla), midbrain, hippocampus, striatum and cortex.

- Metal analysis, as determined by atomic absorption spectroscopy (AAS), has been completed for each brain region.

- Analysis of specific regions of interest (ROIs) from the MR images suggests that brain Mn accumulation is saturated by week 5.

- Comparison of metal content (AAS) to specific ROI R_1 values (a rate constant associated with T_1-weighted images) suggests a regionally specific relationship between brain Mn accumulation and MR parameters.

- Mn brain deposition was shown to be affected by brain Fe levels.

- A model was developed to assess the relationship between brain Mn and Fe deposition

- The effects of Mn and Fe on rat behavior were assessed.

Reportable Outcomes

- Manuscript:
    - Fitsanakis VA, Finkelstein Y, Aschner M. Changes in dietary iron levels affect brain manganese accumulation and distribution. Crete Meting. Cell Biol Toxicol2008; in press 2008.
    - Aschner M, dos Santos APM, Erikson KM, Zheng W. Manganese transport into the brain: putative mechanisms. Corsica Meeting. Metal Ions Biol Med 2008; in press 2008.
    - Zhang N, Fitsanakis VA, Erikson KM, Aschner M, Avison, MJ, Gore JC. A Model for the analysis of competitive relaxation effects of manganese and iron in vivo. NMR Biomed J 2008; in press pending revision.
    - Aschner M, Dos Santos, APM, KM Erikson, Zheng W. Manganese transport into the brain: possible mechanisms. Metal Ions Biol Med 2008; in press.
    - Finkelstein Y, Milatovic D, Aschner M. Modulation of cholinergic systems by manganese. Neurotoxicology 2007; 28:1003-1014.
    - Aschner M, Guilarte TR, Schneider JS, Zheng W. Manganese: Recent advances in its transport and neurotoxicity. Toxicol Appl Pharmacol 2007; 221:131-47.
    - Aschner M, Dorman DC. Manganese: Pharmacokinetics and molecular mechanisms of brain uptake. Toxicol Rev 2007; 25:147-154
    - Fitsanakis VA, Thompson KN, Deery SE, Milatovic D, Shihabi ZK, Brown RW, Aschner M. A chronic iron-deficient/high-manganese diet in rodents results in increased brain oxidative stress and behavioral deficits in the Morris water maze. Neurotoxicity Res 2008; submitted October 2007.
    - Fitsanakis VA, Zhang N, Anderson JG, Erikson KM, Gore JC, Aschner M. Measuring brain manganese and iron accumulation in rats following 14-weeks of low-dose manganese treatment using atomic absorption spectroscopy (AAS) and magnetic resonance imaging (MRI). Toxicol Sci 2008; in press.
    - Fitsanakis VA, Zhang N, Avison MJ, Gore JC, Aschner JL, Aschner M. The use of magnetic resonance imaging (MRI) in the study of manganese neurotoxicity. Neurotoxicology 2006; 27: 798-806.

Current faculty receiving support from the grant:

- Michael Aschner, PhD
- Dejan Milatovic, PhD

References

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AWARD NUMBER: W81XWH-05-1-0239

TITLE: Biomarkers of Early Onset of Manganese Neurotoxicities among Occupationally Exposed Chinese Workers

PRINCIPAL INVESTIGATOR: Wei Zheng, Ph.D.

CONTRACTING ORGANIZATION: Purdue University School of Health Sciences
West Lafayette, IN 47907

REPORT DATE: January 15, 2008

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Biomarkers of Early Onset of Manganese Neurotoxicities among Occupationally Exposed Chinese Workers

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Wei Zheng, PhD

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14. ABSTRACT

The purpose of this cross-sectional study was to establish a distinct value that allows to distinguish Mn-exposed subjects from the general, Mn-unexposed healthy population. Mn-exposed ferroalloy smelters (n=95), power distributing and office workers (122), and unexposed control subjects (106) were recruited to the high, low and control group, respectively. Mn concentrations in saliva, plasma, erythrocytes, urine, and hair were significantly higher in both exposure groups than those in controls. The Fe concentration in plasma and erythrocytes, however, was significantly lower in Mn-exposed workers than in controls. A concept of Mn/Fe ratio (MIR) was then developed. The MIR for erythrocytes (eMIR) and plasma (pMIR) exhibited a significant exposure-group related increase. Linear regression revealed that the airborne inhalable Mn level was significantly associated with eMIR and pMIR. Among all determinants, only eMIR and pMIR were significantly associated with smelter's years of employment. The cut-off value (COV), above which workers are considered to be Mn exposed, was established. At the eMIR COV of 8.8, about 88% of the high exposure smelters had an eMIR above the COV, while 87% of controls had an eMIR below the COV. Taken together, this study suggests that chronic occupational exposure to Mn in smelters increases Mn and decreases Fe in plasma/erythrocytes, respectively. A cut-off eMIR value 8.8 may be useful for assessment of Mn exposure in general populations.

15. SUBJECT TERMS

Manganese, neurotoxicology, biomarker, iron metabolism, manganese mining, human study

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Introduction

The early onset of Mn intoxication is usually subtle and progressive. The initial signs may be categorized as the nonspecific neurological manifestations, psychiatric symptoms, and extrapyramidal signs. The exposed workers may complaint asthenia, anorexia, apathy, insomnia or drowsiness, malaise, somnolence, or diminished libido or impotence. Psychiatric symptoms are more specifically indicative of Mn toxicity, including disorientation, emotional instability, compulsive acts, hallucinations, illusions, delusions, and slurring and stuttering speech with diminished voice. These are followed by selective extrapyramidal disorders such as imbalance in walking or on arising, finger coordination, and tremor.

Since Mn induced neuronal damage is irreversible, the early diagnosis becomes crucial for prevention of Mn toxicity in occupational and environmental exposure scenarios. Based on a recent study on 97 welders in Beijing, China, Dr. Zheng and his colleagues in China has found that serum concentrations of ferritin and transferrin were increased among welders, while serum transferrin receptor levels were significantly decreased in comparison to controls. Moreover, this group found that serum transferrin receptor levels were inversely associated with serum manganese concentrations ($p<0.05$). Thus, these iron regulatory proteins along with Fe itself may serve as the potential useful biomarker for early diagnosis of Mn toxicity.

We propose a three-phase, three-year, cross-sectional study to test the hypothesis that occupational exposure to airborne Mn is associated with health disorders among exposed workers in a time-dose dependent manner. More specifically, we aim to see if airborne Mn levels are positively correlated with levels of Mn in blood, urine, saliva, or hair, and Fe or Fe regulatory proteins in serum, one of which can be used as the biomarker to assess Mn exposure. In addition, we aim to study whether Mn concentrations in biological matrices (blood, urine, or hair) are associated with early signs of health disorders among exposed workers.

In Phase I, the principal task is to further characterize the study sites and to conduct exposure assessment in the environment from which the study subjects will be recruited. During this period, the instruments for air sampling, questionnaires for epidemiological study, documents for data storage, and methodology for laboratory assays (AAS) for Mn, etc., shall be fully prepared or developed.

The Phase II aims to study the biological outcomes of exposure. We will collect biological samples, conduct physical examinations, and determine Mn and biomarker concentrations. Biological samples from all workers will be obtained at the time of physical examination within 10-12 months. The time frame for data collection will be approximately 12 months and the lab analyses will take longer time.

In Phase III, we will put much our effort on statistical analysis to draw the conclusions on our hypotheses. We estimate a 9-12 month period, for we may revisit some of the sampling spots or subjects to verify the data.

Body of Progress Report

1. Human study logistics

The IRB protocol entitled “Biomarkers of Early Onset of Manganese Neurotoxicities among Occupationally Exposed Chinese Workers” (Ref#04-655) was re-approved by the Committee on the Use of Human Research Subjects, Institutional Review Board of Purdue University, on 12 July 2007.

The initial application for IRB approval was sent to the Human Subjects Research Review Board (HSRRB) of the U.S. Army Medical Research and Materiel Command (AMRMC) on 26 Aug 2005. The application was suggested for full review and subsequently reviewed by AMRMC HSRRB on 12 Oct 2005. The protocol was approved by the Committee on 22 Feb 2006. The human research assurance number was approved and granted to the ZMC by U.S. DHHS on 13 Jan 2006. The protocol was re-approved on 19 Dec 2006 and Dec, 2007.

2. Trips to Zunyi City to monitor the study progress

The first trip to Zunyi city was made between April 4-9, 2005. Drs. Zheng (team leader and neurotoxicologist), Rosenthal (expert in exposure assessment), and McGlothlin (expert in industrial hygiene and epidemiology) at Purdue and Dr. Jie Liu of NIH/NCI (expert in bioassays) joined the visit. The purpose was (1) to consolidate working relationship with Chinese counterpart, (2) to establish the direct communication channels between the investigators from the US and China, (3) to clearly define and assign the responsibility to each researcher in this multination team, (4) to train the researchers on the site for how to use the equipment we brought to ZMC, and (5) to discover the potential problems and to solve them on the site. The visit resulted in a signed Research Agreement between Purdue University and ZMC.

The second trip to Zunyi was made between April 17-20, 2006. Dr. Michael Aschner, the Program Director, Dr. Wei Zheng, the PI of this project, and Mr. Dallas Cowan, doctoral student in Zheng group, participated in this site visit. The tasks were for Dr. Aschner to meet the research team and to oversee the progress (Aschner, Zheng), to examine if the human research conduct follows the IRB and other protocols (Zheng, Aschner), to conduct neurobehavioral testing on the subjects (Zheng, Cowan), to monitor laboratory experiments and assays (Zheng, Cowan), to bring some biological samples back to the US for quality control (Cowan), and to discuss the exchange scholar for training propose (Aschner, Zheng). During the trip, six subjects were recruited to the research center. Mr. Cowan trained the researchers for neurobehavioral test, and Dr. Zheng supervised the administration of questionnaires, physical examination, and obtaining biological samples (blood, saliva and hair).

The third trip to Zunyi was made between Nov 10-14, 2006. Dr. Zheng performed the on-site inspection of data storage, confidentiality compliance, and analytical quality control. Dr. Zheng also had the meeting with Chinese team to discuss the progress of the project, technical help needed for sample analysis, and financial issues. During the meeting, the issue was raised on the underestimation of the budget for reagents, consumables, and effort compensation.

The forth trip to Zunyi was made between Oct 30-Nov 3, 2007. Dr. Zheng and Dr. Aschner visited the ZMC, listened the report by Dr. Qiyuan Fan, inspected all clinical and laboratory records, and checked the storage of all biological samples. Dr. Aschner expressed his expectation on this important human research. Dr. Zheng reported the initial data analysis on all subjects, the problems encountered, the solution sought and the time line for the final phase of this research. Prof. Jingshan Shi, the President of ZMC and Dr. Chen, Director of Quizhou Institute of Occupational Safety and Health, met with Drs. Zheng and Aschner. During the meeting, the next phase of MRS study was discussed and planned.

3. All samples by January 2008

We have recruited a total of 323 subjects, who were allocated to each study group based on their job category. The demographic data are presented in Table 1. Among subjects recruited (n=106, 122, and 95 for control, low and high exposed workers, respectively), occupational Mn exposure was determined using personal and area sampling methods; subjects were randomly selected, based on airborne Mn levels and job classification, to control group (airborne Mn <0.003 mg/m^3), low-exposed group (<0.026 mg/m^3), or high-exposed group (0.177 mg/m^3). Following physical examination, the blood, urine, saliva, and hair samples from all subjects were obtained to determine Mn and Fe levels by AAS.

Table 2 summarizes the data on metal concentrations in all biological matrices. Mn concentrations in the whole blood (WB), plasma, urine, hair, and saliva in Mn-exposed workers (both low and high) were significantly higher than those in controls ($p<0.05$) (Table 2). Mn in hair was particularly high; the possibility of external contamination cannot be completely excluded.

Table 3 lists the outcomes of linear regression analyses of the data within each exposure group or the data from all three groups in combination. Mn concentrations in plasma, erythrocytes, and hair did not statistically significantly change as the function of workers' employment year in the job, nor were they associated with workers' age or gender. MnS was weakly, yet significantly, associated with employment year and age ($r=0.12$, $p<0.05$) (Table 3). Mn concentrations in all biological matrices were further analyzed by stratifying for years of employment as <5, between 5-10, and > 10 years. No employment year-associated increase in Mn levels was found in any of these three groups and in any biological matrices (data not shown). When the personal air sampling data (n=43) were paired for linear regression analysis, the airborne inhalable Mn level was found statistically significantly associated with MnS ($r=0.75$, $p<0.01$) and MnE ($r=0.52$, $p<0.01$).

Analysis of Fe concentrations in biological matrices indicated that Fe concentrations in saliva and urine increased (Table 2). However, Fe concentrations in plasma and erythrocytes were significantly reduced, reflecting a general deficiency in Fe among Mn-exposed workers. This is important for establishment of Mn-Fe ratio for Mn exposure. Among Fe regulatory proteins analyzed (ferritin, transferrin and TIBC in serum, ferritin, transferrin receptor, and TIBC in saliva), serum transferrin level was significantly increased among high-exposed workers (26%, $p<0.05$) (Table 4). In general, Mn exposure appeared to affect Fe-regulatory proteins to a less significant extent as it did to metal concentration.

Since Mn exposure resulted in a general increase in Mn levels in major biological matrices and a general decrease in Fe levels in blood compartment, it became appealing to investigate the ratio of Mn and Fe concentrations (MIR) within a given matrix. Since the numerator reflects Mn exposure and the denominator indicates a biological alteration, the MIR would be expected to yield a novel and more sensitive measure of Mn exposure. As an example, the MIR in erythrocytes can be calculated as follows:

$$eMIR = \frac{MnE(\mu g/L)}{FeE(\mu g/L)} \cdot 1000$$

$eMIR$: Erythrocyte Manganese-Iron Ratio

MnE: Mn concentration in erythrocytes

FeE: Fe concentration in erythrocytes

The values for all biological matrices were calculated for each study subject and the statistical data are presented in Table 5.

The MIR for erythrocytes (eMIR) and plasma (pMIR) exhibited a significant increase in smelters compared to controls ($p<0.05$, Table 5). The eMIR of low and high exposure groups represents a respective 126% and 349% increase over eMIR calculated for controls. A similar exposure group-related increase was also observed for pMIR, but the pMIR values between the low and high exposure group were not significantly different. No significant differences were observed for sMIR. The MIR for urine, while also greatly increased, did not show an exposure group-related effect. Similar to MnS, MnE and MnP, the MIR values were significantly associated with airborne inhalable Mn concentrations eMIR ($r=0.70$, $p<0.01$) and for pMIR ($r=0.77$, $p<0.01$).

To be a reasonable indicator of Mn exposure, ideally the biological measures should have (1) external exposure-dose related changes, (2) large, stepwise percentage increases between adjacent groups, and (3) a reasonable threshold above which the sensitivity and specificity of the indicator relative to the exposure can be maximized. Among Mn, Fe and MIR determinants, the parameters such as MnS, MnP, MnE, pMIR, and eMIR had a dose-related increase (Table 2 & 5). The percentage of the increases between adjacent groups is high in MnS, MnP, MnE, pMIR, and eMIR. Thus, these five parameters were chosen for further stepwise multiple regression analysis.

The generalized linear model (GLM) was utilized to determine associations between obtained parameters and the independent variables, including exposure group, years of employment, age, sex and income (Table 6). The Model I reveled that there existed a significant group difference for all five parameters. With addition of years of employment and sex to the model (Model V), eMIR, pMIR or MnP was either significantly or closely associated with worker's years of employment; this relationship, however, was not evident for MnS and MnE. When all of the variables were taken into account (Model VII), only eMIR and pMIR were significantly associated with years of employment.

The method of analysis of covariance (ANCOVA) by controlling one factor level variable and one continuous variable was used to compare between eMIR and pMIR. A post–hoc analysis using Tukey's pair-wise comparison revealed that eMIR was statistically different between each group by controlling all independent variables (exposure group, years of employment, age and sex) (Table 7A). However, the same post-hoc test showed that the pMIR was not statistically different between the low- and high- exposure groups ($p=0.121$) (Table 7B).

As the eMIR appeared to be a promising indicator for Mn exposure, we further used the receiver-operator characteristic (ROC) analysis to establish the threshold value to maximize the sensitivity and specificity of eMIR to Mn exposure. The ROC analysis yield an eMIR value of 8.8 for comparison between the control and high exposure groups, which represents the level where sensitivity and specificity were maximized at 87% each (Fig. 5A). Using the cut-off value (COV) of 8.8, 80 out of 92 control subjects had an eMIR below this COV (87% of controls). Of 83 smelters in the high exposure group, 73 of them had an eMIR above 8.8 (88% of the high exposure smelters). When the subjects in low- and high- groups were combined, the optimal eMIR value with the best sensitivity and specificity (78% each) was 9.68. By using a COV of 9.7, 108 out of 196 total smelters studied (55%) had the eMIR significantly higher than the COV, whereas 89% of control eMIR values were below this COV. By using the similar approach, the pMIR yielded a poorer percentage of workers either above or below the COV in each category than did the eMIR (Table 8). Thus, it appeared that the eMIR may be useful for indicating Mn exposure.

Key Research Accomplishments

- For the first time in literature, we proposed biological measurable values that may truly reflect Mn exposure status in humans. These values (i.e., eMIR and pMIR) are a composite of the blood index of Mn exposure and the biological consequence of such an exposure. It may be useful for clinical diagnosis of Mn intoxication as well as for risk assessment of Mn toxicity in general population.

- This study has been successfully accomplished with regards to the subject recruitment and examination, exposure monitoring, laboratory sample analysis, and data record, entry and analysis. We are in the final stage of statistical analysis and manuscript preparation.

- Local Chinese researchers have been trained along with the progress of this project. They have now had a better sense on the quality of data collection, proper conduct of human study, respect of subject's privacy, and scientific and objective interpretation of data. Data safety monitoring meets the strict guideline of DoD requirement.

Reportable Outcomes

- Abstracts already presented:
  Yes

- Current faculty receiving support from the grant:
  - Wei Zheng, PhD
  - Frank Rosenthal, PhD

- Current students receiving training from participation on projects related to this grant:
  - Dallas Cowan

Conclusions

More than 15 parameters in saliva, plasma, erythrocytes, urine and hair were examined for their utility as a biomarker for Mn exposure. Mn concentrations in all biological matrices were significantly elevated in smelters as compared to control workers. The concentrations of Fe and Fe metabolic proteins in these matrices were also significantly altered to various degrees. The concept of Mn/Fe ratio (MIR) in biological matrices was developed and tested for its applicability to assess Mn exposure. The erythrocyte MIR (eMIR) exhibits good correlations with worker's employment years and airborne Mn levels. A cut-off eMIR value 9.0 may be useful for assessment of Mn exposure in general populations.

References

1. Cowan, DM, Fan, QY, Shi, XJ, Zou, Y, Rosenthal, FS, Aschner, M, and Zheng, W (2007). Manganese (Mn) in saliva as an indicator for occupational exposure in Chinese smelting workers. Abstract to 2007-SOT meeting in Charlotte, NC.

Manganese (Mn) in saliva as an indicator for occupational exposure in Chinese smelting workers.
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Exposure to Mn occurs in both environmental and occupational settings, leading to various degrees of neurotoxicity. This cross-sectional study was designed to test the hypothesis that occupational exposure to airborne Mn among active smelting workers was associated with elevated metal concentrations, i.e., Mn and iron (Fe), in body fluids. Our goal is to investigate 100 subjects each in control, low- and high- Mn exposed workers. The cohort is located in Zunyi City, Southwest China. We have completed about 23-40% of sample collections (n=42, 23, and 30 for control, low and high exposed workers, respectively). Occupational Mn exposure was determined using personal and area sampling methods; subjects were randomly selected,

based on airborne Mn levels and job classification, to control group (airborne Mn <0.01 mg/m^3), low-exposed group (<0.1 mg/m^3), or high-exposed group (>0.3 mg/m^3). Following physical examination, the blood, urine, saliva, and hair samples from all subjects were obtained to determine Mn and Fe levels by AAS. No age difference was identified among the 3 study groups. Mn concentrations in the whole blood (WB), plasma, urine, hair, and saliva in high-exposed workers were significantly higher than those in controls ($p<0.05$). Among low-exposed workers, significant differences were observed only in the WB, plasma, and hair. The increase in Mn levels in body fluids appeared to be associated with airborne Mn levels except for plasma Mn. Linear regression analyses revealed that saliva Mn concentration was significantly associated with years of employment ($r=0.40$, $p<0.05$). Interestingly, Mn saliva level was also significantly correlated with Mn WB concentrations. ($r=0.32$, $p<0.05$). Additionally, we found that Fe concentrations in saliva and urine were significantly higher in high-exposed workers than in controls ($p<0.05$). These preliminary data indicate that among active smelting workers, Mn levels in body fluids reflect environmental Mn exposure. Mn concentrations in saliva may represent a useful non-invasive indicator of Mn exposure. (Support in part by NIEHS ES 08164 and DoD USAMRMC W81XWH-05-1-0239).

2. Cowan DM, Fan QY, Zou Y, Shi XJ, Rosenthal FS, Aschner M, and Zheng W (2008). Manganese Exposure in 328 Smelting Workers: Relationship among External/ Internal Markers and Neurological/ Psychomotor Examinations. Platform Presentation in 2008 SOT meeting in Seattle.

Manganese Exposure in 328 Smelting Workers: Relationship among External/ Internal Markers and Neurological/ Psychomotor Examinations.

Dallas M. Cowan[1], Qiyuan Fan[2], Yan Zou[2], Xiujuan Shi[3], Frank S. Rosenthal[1], Michael Aschner[4], and Wei Zheng[1*]
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Reliable biomarkers for Mn exposure are not available at present. The purpose of this cross-sectional study was to explore the relationship between airborne Mn levels (MnAir) and changes in biological and neurological parameters among smelters who are exposed to Mn in manufacturing ferroalloys. Chinese workers representing control, low and high exposures were recruited with each group consisting of 110, 104, and 114 subjects, respectively. The airborne Mn levels were <0.04 mg/m^3, 0.04-0.1 mg/m^3 and >0.1 mg/m^3 for control, low and high exposure groups, respectively. Mn levels in serum, red blood cells (MnRBC), saliva (MnSa) and hair were significantly higher in Mn-exposed subjects than controls. Linear regression revealed that MnRBC and MnSa were significantly associated with MnAir ($r=0.71$, $p=0.02$ for MnRBC; $r=0.58$, $p=0.025$ for MnSa). Interestingly, when the ratio of Mn/Fe in RBC from each subject was used for analysis, the mean Mn/Fe ratios (0.47±SD0.46, 0.93±0.39 and 2.02±1.26 in control, low and high exposure groups, respectively) represented a respective 98% and 345% increase in Mn-exposed subjects, percentages that are much greater than those using MnRBC or MnSa alone. The Mn/Fe ratio in RBC was also significantly correlated with the inhalable MnAir ($r = 0.56$, $p<0.01$). Additionally, analysis of movement dysfunction using the Purdue Pegboard, indicated that highly exposed workers ≥ 40 years of age produced scores that were significantly lower than 1) control subjects who were in the same age group or 2) high Mn exposed smelters < 40 years of age. Taken together, our data suggest that smelting workers are at risk when exposed to airborne Mn. The ratio of Mn/Fe in RBC may be a better estimate for Mn exposure assessment (NIEHS ES 08164 and DoD USAMRMC W81XWH-05-1-0239).

Table 1. Summary of demographic information

		Mn Exposure Groups	
	Control	Low	High
N	106	122	95
men	86	84	89
women	20	38	6
Age	37.7 ± 7.37 (18-56)	34.5 ± 6.89* (20-52)	35.4 ± 6.69 (22-53)
Employment years in current job	2.61 ± 1.66 (0.5-6)	5.03 ± 3.21* (0.1-12)	4.11 ± 2.81* (0.1-12)
Distance from home to work (km)	3.97 ± 0.60 (0-25)	1.44 ± 0.20* (0-20)	2.07 ± 2.44* (0-10)
Inhalable Mn level (mg/m^3)	0.003 ± 0.009 (0.00-0.04)	0.026 ± 0.028* (0.01-0.11)	0.177 ± 0.103**(0.098-0.374)

Data represent mean ± S.D. (range). *p<0.05 compared to control **p<0.05 compared to control and low exposure;

Table 2. Metal concentrations in biological matrices

Biological Matrices		Mn Exposure Groups			
	Control	Low	Low %change	High	High %change
Mn Concentration					
Saliva (µg/L)	9.98 ± 6.10	22.3 ± 11.3*	+123	31.3 ± 13.6**	+214
Plasma (µg/L)	9.97 ± 7.97	23.3 ± 19.2*	+134	30.4 ± 19.5**	+205
Erythrocytes (µg/L)	4.68 ± 3.59	7.45 ± 6.15*	+60	15.6 ± 9.01**	+233
Urine (µg/L)	0.73 ± 0.35	3.09 + 1.67*	+323	2.46 ± 1.53**	+237
Hair (mg/kg)	1.51 ± 2.00	32.1 ± 29.2*	+2026	37.6 ± 22.5*	+2390
Fe Concentration					
Saliva (µg/L)	778 ± 445	1385 ± 774*	+78	1405 ± 826**	+81
Plasma (mg/L)	8.98 ± 4.85	5.33 ± 3.14*	-41	6.93 ± 4.80**	-23
Erythrocytes (mg/L)	1091 ± 455	750 ± 150*	-31	777 ± 109*	-29
Urine (mg/L)	0.78 ± 0.31	0.74 ± 0.42	-5	0.60 ± 0.43**	-23
Hair (mg/kg)	11.5 ± 15.7	35.9 ± 25.7*	+212	35.0 ± 29.0*	+204

Data represent mean ± S.D. *p<0.05 compared to control. **p<0.05 compared to both control and low exposure group
%change: exposure group compared to control (exposed concentration-control concentration/control concentration · 100)

Table 3: Correlation matrix

	MnS	MnP	MnE	MnU	MnH	sMIR	pMIR	eMIR	Age	Years
MnS	-	0.27**	0.36**	0.31**	0.35**	0.43**	0.22**	0.43**	0.18**	0.12*
MnP	-	-	0.25**	0.08	0.32**	0.17**	0.84**	0.23**	-0.13*	0.01
Mn-E	-	-	-	0.11	0.26**	0.10	0.19**	0.94**	-0.08	0.00
MnU	-	-	-	-	0.29**	0.12	0.17**	0.16**	-0.08	0.16**
MnH	-	-	-	-	-	0.16**	0.27**	0.24**	-0.06	0.01
vMIR	-	-	-	-	-	-	0.07	0.12	-0.05	-0.06
pMIR	-	-	-	-	-	-	-	0.19**	-0.12	0.01
eMIR	-	-	-	-	-	-	-	-	-0.12	0.00
Age	-	-	-	-	-	-	-	-	-	0.10
Years of Employment	-	-	-	-	-	-	-	-	-	-

Table 4: Fe-regulatory protein concentration within biological matrices

	Mn Exposure Groups				
Iron Regulatory Protein	Control	Low	Low %change	High	High %change
Serum Ferritin (ng/mL)	31.8 ± 21.6	36.3 ± 24.5	+14	35.5 ± 27.8	+12
Serum Transferrin (g/L)	1.65 ± 0.45	1.97 ± 0.36	+19	2.08 ± 0.33*	+26
Serum TIBC (µmol/L)	25.0 ± 5.97	20.8 ± 3.13*	-17	22.7 ± 2.89**	-9
Saliva Ferritin (ng/mL)	24.0 ± 52.7	9.15 ± 36.8	-62	12.1 ± 29.3	-50
Saliva Transferrin Receptor (µg/mL)	18765 ± 19760	13432 ± 16486	-28	29034 ± 21452	+55
Saliva TIBC (µmol/L)	4.61 ± 3.84	2.17 ± 3.54*	-53	4.13 ± 5.61	-10

Data represent mean ± S.D. *p<0.05 compared to control. **p<0.05 compared to both control and low exposure group

Table 5: Manganese-Iron ratio (MIR) in biological matrices

	Mn Exposure Groups				
Biological Matrices	Control	Low	Low % Change	High	High % Change
Saliva (x100)	1.92 ± 2.24	2.19 ± 1.98	+14	2.92 ± 2.16	+52
Plasma	1.52 ± 1.38	4.52 ± 3.31*	+197	5.03 ± 3.50*	+231
Erythrocytes (x1000)	4.65 ± 3.90	10.5 ± 8.76*	+126	20.9 ± 12.9**	+349
Urine	1.04 ± 0.56	7.65 ± 9.33*	+636	5.90 + 5.26*	+467
Hair	0.13 ± 0.11	0.87 ± 0.65*	+569	1.37 ± 0.89**	+954

Data represent mean ± S.D. *p<0.05 compared to control. **p<0.05 compared to both control and low exposure group. Significance confirmed with Tukey's Post-hoc analysis

Table 6. Changes of biomarkers as the function of exposure, years of employment, age and sex by generalized linear model (GLM) analysis

		eMIR			pMIR			MnP			MnS			MnE		
Models	Variables	F-Statistic	P	Adjusted R^2	F-Statistic	P	Adjusted R^2	F-Statistic	P	Adjusted R^2	F-Statistic	P	Adjusted R^2	F-Statistic	P	Adjusted R^2
Model I	Exposure Group	70.98	0.000	32.78	35.42	0.000	20.08	34.43	0.000	18.38	89.20	0.000	38.15	70.58	0.000	31.62
Model II	Exposure Group	68.83	0.000	32.95	32.44	0.000	19.61	31.73	0.000	18.47	85.27	0.000	39.03	40.05	0.000	31.83
	Age	0.52	0.472		0.35	0.553		1.38	0.241		4.75	0.030		0.06	0.811	
Model III	Exposure Group	68.35	0.000	32.79	35.70	0.000	20.65	34.77	0.000	18.93	75.49	0.000	36.11	68.74	0.000	31.91
	Years Empl.	2.68	0.103		4.84	0.029		3.59	0.059		0.15	0.696		1.95	0.164	
Model IV	Exposure Group	66.35	0.000	32.94	66.35	0.000	32.94	31.53	0.000	18.87	70.83	0.000	36.83	68.45	0.000	32.18
	Years Empl.	2.77	0.097		2.77	0.097		2.78	0.097		0.01	0.935		2.29	0.132	
	Age	0.12	0.724		0.12	0.724		0.81	0.368		3.67	0.056		0.00	0.946	
Model V	Exposure Group	72.70	0.000	34.17	36.16	0.000	21.52	35.07	0.000	18.87	78.33	0.000	36.85	68.59	0.000	31.90
	Years Empl	3.5	0.063		5.59	0.019		3.82	0.052		0.37	0.544		2.22	0.138	
	sex	6.68	0.010		3.90	0.049		0.79	0.376		4.14	0.043		0.97	0.326	
Model VI	Exposure Group	70.84	0.000	34.29	33.50	0.000	20.93	31.72	0.000	18.74	72.70	0.000	37.26	67.94	0.000	32.18
	Years Empl.	3.87	0.050		5.41	0.021		3.02	0.084		0.10	0.748		2.65	0.104	
	Age	0.01	0.907		0.02	0.889		0.53	0.467		2.40	0.122		0.006	0.813	
	Sex	6.48	0.011		3.79	0.053		0.54	0.465		2.85	0.092		1.00	0.318	
Model VII	Exposure Group	63.00	0.000	33.30	27.66	0.000	18.86	27.62	0.000	17.30	61.13	0.000	34.89	44.80	0.000	36.39
	Years Empl.	4.80	0.029		4.62	0.033		1.82	0.179		0.09	0.760		3.01	0.085	
	Age	0.05	0.817		0.000	0.973		0.28	0.599		1.55	0.215		1.05	0.398	
	Sex	6.47	0.012		3.04	0.083		0.64	0.423		2.63	0.106		0.02	0.895	
	income	0.27	0.607		0.000	0.989		0.48	0.487		0.22	0.636		1.49	0.043	

Table 7A: Pair-wise comparison of Model VI by Tukey (ANCOVA one continuous variable for eMIR)

Model	Variables		Coefficient	Test Statistic	P	
Model III	Group		-	F=76.8	0	
eMIR	Years of Employment		-0.404	t=-2.02	0.045	
			Difference of Means	SE of Difference	t-statistic	Adjusted P
Pairwise	Control	Low	-5.78	1.46	-3.95	0.002
Comparison	Control	High	-16.6	1.4	-11.82	0.000
by Tukey	Low	High	-10.8	1.3	-8.31	0.000

Table 7B: Pair-wise comparison of Model VI by Tukey (ANCOVA one continuous variable for pMIR)

Model	Variables		Coefficient	Test Statistic	P	
Model III	Group		-	F=38.3	0.000	
pMIR	Years of Employment			t=-2.19	0.029	
			Difference of Means	SE of Difference	t-statistic	Adjusted P
Pairwise	Control	Low	-3.22	0.5	-6.411	0.000
Comparison	Control	High	-4.075	0.47	-8.622	0.000
by Tukey	Low	High	-0.853	0.43	-1.964	0.121

Table 8: Cut-off value (COV) Comparison for MIR

MIR	Group Comparison	COV	# above COV /Total (High)	# above COV/Total (Low and High)	# below COV/Total (control)
eMIR	Control Vs High	8.80	73/83 (88%)	124/196 (63%)	80/92 (87%)
	Control Vs Low and High	9.68	69/83 (83%)	108/196 (55%)	82/92 (89%)
pMIR	Control Vs High	2.74	67/89 (75%)	133/193 (69%)	69/82 (84%)
	Control Vs Low and High	3.27	60/89 (67%)	114/193 (59%)	75/82 (91%)

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Chair & Coordinator of the Mn Health Research Program Steering Committee & Administrator of the Research Activity Awareness Services

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Anne Tremblay

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14. Abstract

The primary activities of the MHRP Steering Committee are to select the projects to be included in the MHRP and to review their progress. The role of the Chair and Coordinator of the Steering Committee is to ensure that the projects selected are of irreproachable scientific worth, but also take into account the primary concern of the industry and the US Department of Defense: preserving the health of their workers. The composition of the Steering Committee (a mix of academics, scientists, and qualified industry representatives), along with the active participation of the program's principal investigator, Dr. Michael Aschner, ensure that these goals are being met.
Administering the Research Activity Awareness Services (RAAS) implies working in tandem with Dr. Leonard Levy and his team so that his RAAS project is made available on a MHRP-dedicated web site: www.manganese-health.org The web site, launched in Feb. 06, contains information about the MHRP, manganese, along with useful contacts and news.

15. SUBJECT TERMS
Manganese, manganese health, neurotoxicology, iron deficiency, welding, manganese mining, nutrition.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Anne Tremblay
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)* +33(0)1 45 63 06 34

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Introduction, Body and Key Accomplishments

Chair & Coordinator of the Mn Health Research Program Steering Committee

The MHRP Steering Committee is Chaired by Anne Tremblay, Secretary General of the International Manganese Institute (IMnI). Its members in 2007 were Dr. Barbara Beck of Gradient Corporation, Dr. Tomas Guilarte of the Johns Hopkins University, Dr. Joan Cranmer of the University of Arkansas, Dr. Leonard Levy, Cranfield University (UK), Dr. Harry Roels, Professor Emeritus at the Catholic University of Louvain (Belgium), Dr. W Les Dees, Professor at Texas A & M University, Dr. Jerry Roper of Afton Chemicals, Mr. Pierre Rousseau of Eramet, Mr. Dirk van Niekerk of BHP Billiton, and Mr. John Hilbert of Kinghorn, Hilbert & Associates. The Steering Committee works in close tandem with Dr. Michael Aschner, Professor at Vanderbilt University and the Principal investigator for the entire program.

The MHRP Steering Committee met three times in 2007.
The 1st meeting was held on May 8th in Washington to review the Budget and the progress being made on the different projects. At this meeting, the Steering Committee determined that there sufficient funds left in the budget to cover a 3rd Phase of the MHRP.

A call for pre-proposals was launched in the summer and 42 letters of intent (LoI) for Phase 3 were received. The Committee met by telecon on October 16, to select those proposals that merited further consideration. Twenty of the original 42 were selected and these researchers were requested to submit full proposals. On December 19, the Steering Committee met to review the 20 full proposals. After much deliberation, the Committee decided that the entire 20 could be subsidized, if each of the project leaders was willing to cut his budget by 10%. Dr. Aschner was commissioned to contact each project leader to make this request.

Tracking Welding Issues for the MHRP

Manganese is a component of coated welding rods and various steel alloys. As a result, there can be significant exposures to a finely divided dust/fume in welding operations, and massive exposures which have been associated with a debilitating neurological disease. Welding is one of the primary industrial activities in defense department activities common to all of the armed forces. For this reason, Anne Tremblay continued to track the litigation cases aiming to prove that Mn in welding rod fumes causes Parkinson's disease.

She maintained regular contact with Charles Read, Senior Partner with O'Melveny & Myers LLP, a law firm with offices in Washington, DC and Los Angeles, which is representing the defendants in many of these cases.

Keeping the Metals Industry Informed of the MHRP

During 2007, Anne Tremblay met with a number of metals associations to inform them of progress on the MHRP. These included: the North American Metals Council, the International Council on Mining and Metals, The International Iron and Steel Institute, Eurometaux and Euroalliages.

Administrator of the Research Activity Awareness Services

The International Manganese Institute is funded to provide an MHRP dedicated web site to house the Research Activity Awareness Services. The site is also designed to include general information about the MHRP, including project descriptions, along with background on manganese and its uses, useful contacts, news & developments.

Reportable Outcomes

The MHRP web site (www.manganese-health.org) was launched on February 10th, 2006.

Throughout 2007, the site was updated regularly. A major update on the progress of the 14 existing research projects was undertaken over the summer and completed in the fall.

Conclusions

The Steering Committee is fully functional. Throughout 2007, it continued to track the 14 MHRP studies underway, besides inaugurating Phase 3.

References

Not applicable

AD_____
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AWARD NUMBER: W81XWH -05-1-0239

TITLE: Manganese Research Health Project (CFDA No. 12.420): Provision of Research Activity Awareness Services

PRINCIPAL INVESTIGATOR: Prof L.S Levy OBE

CONTRACTING ORGANIZATION: Institute of Environment and Health, First Floor, Building 63, Cranfield University, Cranfield, Bedfordshire MK43 0AL, UK

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report contains proprietary information

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14. ABSTRACT

IEH has worked – in collaboration with the International Manganese Institute (IMnI) and their subcontractor Intendance Limited – since November 2005 on provision of knowledge management services (Research Core 6) to provide the research and regulatory communities with web-based information on developments relevant to assessing adverse health effects of manganese. Fields considered comprise: epidemiology; human exposure assessment; toxicological mechanisms; human susceptibility; and treatment of patients exposed to excessive levels of manganese.
To date, 7 reports identifying published information (covering January 2002 to November 2007) have been posted on the MHRP website. The final update report scheduled under the existing contract is scheduled for posting in late January 2008. A Research Overview Report discussing the significance of new knowledge gained through research published between January 2002 and February 2007, was posted on the MHRP website in December 2007, and a second annual report (for March 2007 – February 2008) is scheduled for publication in March 2008. IEH and collaborators also established the Database of Global Research Activity on Manganese (Dogram) as an up-to-date source of ongoing research activities; over 3000 "hits" have been recorded for Dogram since the launch in June 2007 while the MHRP website had a total of 2167 visitors during 2006, and 4,113 visitors during the period 1st January to 1st October 2007.

15. SUBJECT TERMS
Manganese; Health; Published information

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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					Len Levy, PhD

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Introduction

While manganese is an essential trace element, associations with a number of adverse human health effects have been identified at high occupational or environmental exposures, and the health effects of inorganic forms of manganese published before 2002 was comprehensively reviewed in a recent Criteria Document (CD) (IEH, 2004). Since the time of production of the 2002 assessment, there has been - and continues to be - extensive research activity (including that embodied within the current MHRP research program) into aspects as diverse as occupational and environmental exposures, epidemiology, mechanisms of toxicity, and the development and implementation of new medical approaches to the treatment of excessive exposures.

Given the scale of current research (with, for example, over 500 references relating to inorganic forms published in 2002 alone), this project (Research Core 6), undertaken by the Institute of Environment and Health (IEH) at Cranfield University, in collaboration with the International Manganese Institute (IMnI) and their subcontractor Intendance Limited, was designed to provide readily available sources by which researchers and those sponsoring research programmes could readily access up-to-date information on recently reported and ongoing projects, so as to avoid duplication of efforts and thereby proactively enhance efficiency. In addition, the data sources generated in this project were intended to facilitate and encourage multicentre and multidisciplinary research collaborations through facilitating networking and to provide both the technical and general viewer with readily understandable and freely available sources of information on the latest developments in understanding.

BODY

OVERALL OBJECTIVES

The objectives of the Core 6 research project is to:

- create a database of information on recently completed and ongoing research projects;
- provide a current awareness service on a quarterly basis; and
- provide short annual "state of the science" reviews, identifying recent key scientific papers.

The intention has been to provide these services – on a free-access basis – via a specially constructed web-site, containing this and other information relevant to the study of the health effects of manganese.

PROJECT ELEMENT 1 – Establishment of a Database of Information

Following a detailed analysis of functionaility and specification requirements focussing on ease of use and access to the information by users, the project team developed a database and website using Microsft database and Internet server technologies (including .Net, SQL Server, XML). The database, known as *"Database of Global Research Activity on Manganese*" (DOGRAM), is now available on the website at: http://www.manganese-health.org/home. The database was designed to be viewed using Browse facilities (by Project, Researcher, Research organization and Funder) and by use of Search facilities (using a customized 4-level thesaurus based on defined keywords as well as by a free text search facility).

Information for entry onto DOGRAM is gathered by a dedicated project team using email prompts to potential researchers and research groups around the world (identified from the output of the quarterly update report; see below) or through researchers obtain the questionnaire in electronic form and returning it either as hardcopy by post or as an email attachment.

Thus, DOGRAM is a fully searchable inventory of current research activities relating to the potential health effects of, and methods of controlling and treating, exposure to manganese. As such, its scope is intended to encompass investigation of both inorganic and organic forms of manganese, and includes:

- Estimation of the contributions of environmental and occupational manganese exposure to health, disease and dysfunction;
- Investigation of the physiological and biochemical mechanisms (including toxicokinetic considerations);

- Investigation of the physiological mechanisms that govern manganese accumulation within the brain, with special emphasis on the role of olfactory transport of the metal;
- Assessment of the influence of factors, such as age, nutritional deficiencies, pre-existing disease and genetics, that influence individual susceptibility to manganese;
- Investigation of the roles and mechanisms of manganese toxicity, including its role in neurodegenerative disease;
- Measurement and/or modelling of occupational or environmental exposure;
- Identification of existing and novel biomarkers of exposure or adverse effects; and
- Development and implementation of new medical approaches to the treatment of excessive manganese exposure.

DOGRAM thus provides in an ongoing manner an effective and powerful tool for identifying current and recently completed research activities and the key workers in relevant fields. As such, it will be of value to stakeholders and researchers, providing a means of minimizing the risk of duplication of effort, and over time should facilitate the identification of changing patterns of research activity, gaps in programmes, opportunities for collaboration, and emerging areas of concern.

PROJECT ELEMENT 2 – Current Awareness Services

Using a comprehensive structured search strategy (see Tabulations below) literature searches were performed at approximately 13 week intervals on Medline (1966+), Embase (1974+), Pascal (1990+), Biosis (+1969) and Toxfile (1966+), using the host Dialog DataStar. The search terms used to denote for manganese substance (see Set 1), CAS (see Set 2) and toxicity (see Set 3) are listed below.

Set 1

Substance – title, abstract, descriptors
Braunite
Cianciulliite
Ferromanganese or ferro manganese - FeMn
Ferrosiliconmanganese or ferro silicon manganese
Manganese ore$1
Manganese oxide$1
Manganese sulphate or manganese sulphate
Manganese with steel – (title, abstract)
Manganous salt$1
Manganous Manganic Oxide or Hausmannite – Mn_3O_4
Polianite
Pyrochroite
Pyrolusite (manganese oxide)
Ramsdellite (manganese oxide)
Siliconmanganese or silicon manganese
Sodium manganate – Na_2MnO_4
Manganese
Manganese carbonate – $MnCO_3$
Manganese chloride or Manganese (II) chloride – $MnCl_2$
Manganese (III) fluoride – MnF_3
Manganese oxide or Manganese tetroxide – Mn_3O_4
Manganese (II) oxide – MnO
Manganese (III) oxide – Mn_2O_3
Manganese dioxide or Manganese (IV) oxide – MnO_2
Manganese nitrate or Manganese (II) nitrate – $Mn(NO_3)_2$
Manganese sulphate or Manganese (II) sulphate – $MnSO_4$
Manganese sulphide or Manganese (II) sulphide – MnS
Manganese oxide – MnO
Barium manganate – $BaMnO_4$

Potassium manganate – K_2MnO_4
Potassium permanganate or Potassium (VII) manganate – $KMnO_4$

Set 2

CAS No.	Substance
7439-96-5	Manganese
598-62-9	Manganese carbonate – $MnCO_3$
13446-34-9	Manganese chloride tetrahydrate
7773-01-5	Manganese chloride or Manganese (II) chloride – $MnCl_2$
7783-53-1	Manganese (III) fluoride – MnF_3
1317-35-7	Manganese oxide/Manganese tetroxide – Mn_3O_4
1344-43-0	Manganese (II) oxide – MnO
1317-34-6	Manganese (III) oxide – Mn_2O_3
1313-13-9	Manganese dioxide or Manganese (IV) oxide – MnO_2
10377-66-9	Manganese nitrate or Manganese (II) nitrate – $Mn(NO_3)_2$
15710-66-4	Manganese (II) nitrate hydrate
7785-87-7	Manganese sulphate or Manganese (II) sulphate – $MnSO_4$
18820-29-6	Manganese sulphide or Manganese (II) sulphide – MnS
1344-43-0	Manganese oxide – MnO
7787-35-1	Barium manganate - $BaMnO_4$
10294-64-1	Potassium manganate – K_2MnO_4
7722-64-7	Potassium permanganate or Potassium VII manganate -$KMnO_4$

Set 3

Medline, Toxline	**Embase**	**Biosis, Pascal**
Carcinogen$5.ti,de,ab.	Carcinogen$5.ti,de,ab.	Carcinogen$5.ti,de,ab.
Tumor-markers-biological#	Carcinogen-testing#	Mutagen$5.ti,de,ab.
Carcinogenicity-tests#	Carcinogenic-activity#	Genotoxic$5.ti,de,ab.
Carcinogens-environmental#	Carcinogen-dna-interaction#	Cytotox$5.ti,de,ab.
Mutagen$5.ti,de,ab.	Mutagen$5.ti,de,ab.	Epidemiology.ti,de,ab.
Mutagenecity-tests#	Mutagenic-agent#	
Genotoxic$5.ti,de,ab.	Chemical-mutagen#	
dna-damage#	Promutagen#	
Cytotox$5.ti,de,ab.	Mutagen-testing#	
Epidemiologic-factors#	Chemical-mutagenesis#	
Epidemiologic-methods#	Environmental-mutagen#	
Epidemiology#	Mutagenic-activity#	
Effect-modifiers-epidemiolgy#	Genotoxic$5.ti,de,ab.	
Epidemiolog$2.ti,de,ab.	Cytotox$5.ti,de,ab.	
	Cytotoxic-agent#	
	Cell-mediated-cytotoxicity#	
	Cytotoxicity-test#	
	Epidemiology#	
	Cancer-epidemiology#	

The terms/phrases were searched for in abstracts, descriptors and titles; Truncation was used where appropriate.

Set 1 and 2 were then combined using the Boolean operator 'OR', and the results from the Set 1/2 were then combined with Set 3, using the Boolean operator 'AND'.

Based upon this exhaustive search of the published information, relevant English-language papers on manganese were identified and categorized by an experienced toxicologist, before preparation of a summary report which is then posted on the MHRP website. Abstracts are categorized into the following sections:

Section 1 - EXPOSURE MEASUREMENT AND MODELLING: Papers relating to the measurements or modelling of environmental and occupational Mn exposure, the development of biomarkers of exposure or effect.

Section 2 - HEALTH EFFECTS: Papers on the influence of Mn on health, disease and dysfunction.

Section 3 - MECHANISMS: Papers on the physiological, biochemical and cellular mechanisms underlying the toxic effects of Mn.

Section 4 - HUMAN SUSCEPTIBILITY: Papers relating to assessment of the influence of genetic and epigenetic factors on human susceptibility to the effects of Mn.

Section 5 - TREATMENT AND IMAGING: Papers on the development and implementation of new medical approaches to the treatment of excessive Mn exposure.

Section 6 - MISCELLANEOUS: Other papers considered of interest or potential relevance to the study of the health effects of Mn.

To date, 7 reports identifying published information (covering January 2002 to November 2007) have been posted on the MHRP website. The final update report scheduled under the existing contract is scheduled for posting in late January 2008.

PROJECT ELEMENT 3 – Production of state of the science reviews on manganese

A Research Overview Report discussing the significance of new knowledge gained through research published between 2002 (publications before this were addressed in the IEH Criteria Document) and February 2007 (time of publication of the 4th awareness update report) was posted on the MHRP website in December 2007. This report summarised some of the established thinking on the toxicology of manganese and its inorganic compounds, and incorporated some more recently published studies that have appeared in the scientific literature and are identified on the MHRP database. It was prepared for use by a wide readership (including researchers, interested scientists and health professionals) but may also be of value to any laypersons who may wish to have an overview of manganese toxicity and recently published research.

In summary, it was emphasised that manganese is important in the processing of biological processes but does present a potential toxic risk at excess concentrations as it has the ability to disrupt homeostatic mechanisms, especially those which involve elemental iron. Severe manganese neurological toxicity - or manganism - is correlated closely to the symptoms of Parkinson's disease. Although not entirely alike, there is considerable overlap in the biological effects and some of the symptoms observed. Manganese absorption appears most relevant via the inhalation route where particle size and solubility will have an impact on the severity of the metal in the airways. It is rapidly absorbed and distributed by a series of transporter systems, primarily DMT-1, into the brain where it exerts its neurological effects. Oxidation of the divalent cation to its trivalent state also results in chronic neurological effects. The transitions between oxidation states also have an effect on the cellular-related toxicities of manganese.

High doses of manganese cause an increase in glutamate secretion and reduction in dopamine release, actions which are postulated to be direct consequences of manganese toxicity. The production of radicals (such as the superoxide ion) is also relevantly linked to manganese oxidation and subsequent genetic instabilities.

Abnormally high or low levels of manganese are associated with disease states in humans. The evidence base on the pharmacokinetics, transport of the metal, dietary and environmental factors and tissue distribution is increasing but it is still difficult to attempt to establish an accurate assessment of the risk to humans. The development of models for risk assessment is problematic, as there is very little data on non-human primates, and extrapolation of existing animal data to humans is difficult due to interspecies differences. Recent studies, in particular those cited above, have informed on a number of key aspects regarding the mechanisms by which manganese may exert neurological effects, and research is underway that may inform on the situation over the course of the next few years.

A second report (to focus on publications in the period March 2007 - February 2008) is scheduled for publication in March 2008.

Impact

The MHRP website and in particular the database provides information on the objectives, scope and methods being employed in ongoing research projects, together with insight into funders with an interest in this field (information that would not be readily available in a consolidated format through other means). As such this facilitates the development of an understanding of the ongoing profile of research, an aspect that cannot be identified from the existing databases on published literature which are, by their very nature, retrospective, and may be several years following completion of investigations. It is designed to be of value to a wide audience, drawing as it does on a wide rnage of study types of potential relevance to addressing the potential health effects of manganese. These include topic areas such as: occupational and environmental epidemiological studies, clinical case reports, experimental volunteer studies, and *in vivo* and *in vitro* mechanistic studes (including any application of *–omic* technologies). Together with the awreness update reports, produced at 13 week intervals and the overview scientific assessments, this project has succeded in establishing a valuable information resource to researchers, funders and regulators and the wider communittee.

Conclusions

As part of the initial phase of the research programme supported by the Manganese Health Research Program (MHRP), the Institute of Environment and Health (IEH) at Cranfield University, UK, has undertaken — in collaboration with the International Manganese Institute (IMnI) and their subcontractors Intendance Limited, UK (that designed and maintains the MHRP website) — the provision of knowledge management services (identified as Research Core 6: Provision of Research Activity Awareness Services in the MHRP Phase 1 research programme) for the MHRP.

Our collaborative activities have been directed towards providing the MHRP, and the wider research and regulatory community, with easily accessible (web-based) information on the latest developments in the fields of: epidemiology; human exposure assessment; toxicological mechanisms and the study of human susceptibility; and the treatment of patients exposed to excessive levels of manganese (see: http://www.manganese-health.org/home).

The Year 1 activities on the project were initiated at IEH in November 2005 and, with the award of a second year extension, it is anticipated that – if the requested extension agreement is forthcoming– there are sufficient funds available under the existing contract to support IEHs activities throughout the current (second year) round of the awareness service provision (i.e. to March 2008) thus resulting in the eventual overall generation of eight quarterly updates and two annual overviews of the state of the science.

There is already clear evidence of the interest in the services offered on the MHRP website with, during 2006, a total of 2,167 visitors to the site and for the period 1st January to 1st October 2007, already 4,113 visitors recorded for the site. In addition, over 3000 “hits” have been recorded on the Database of Global Research Activity on Manganese (Dogram) since its launch in June 2007.

Key Research Accomplishments

This service focused project has achieved all its objectives, namely:

- Creation of a database (DOGRAM) of information on recently completed and ongoing research projects;
- Provision of a current awareness service of published papers on a 13-week (quarterly) basis; and
- Publication of short annual “state of the science” review reports, identifying recent key scientific papers and findings.

Reportable Outcomes

Creation of a database (DOGRAM) of information on recently completed and ongoing research projects;

Provision of a current awareness service of published papers on a 13-week (quarterly) basis; and

Publication of short annual “state of the science” review reports, identifying recent key scientific papers and findings.

Current faculty receiving support from the grant:

- Lenard Levy OBE, PhD
- Philip Holmes
- Lini Ashdown

References

IEH (2004) *Occupational exposure limits: Criteria document for manganese and inorganic manganese compounds (Web Report W17*), Report by Institute for Environment and Health/ Institute of Occupational Medicine, Published: Leicester, UK, MRC Institute for Environment and Health (Available: http://www.silsoe.cranfield.ac.uk/ieh/publications/publications.html).

AD_____
(Leave blank)

Award Number:
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TITLE:
(Enter title of award)
Development of guidance and tools to facilitate the use of routine exposure data in future epidemiology studies of manganese exposed workers

PRINCIPAL INVESTIGATOR:
(Enter the name and degree of Principal Investigator and any Associates)
Dr Robert J. Aitken

CONTRACTING ORGANIZATION:
(Enter the Name, City, State and Zip Code of the Contracting Organization)
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Edinburgh, UK
EH14 4AP

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Introduction

In the manganese production industry, several companies routinely collect information on exposures in the workplace, and several carry out health surveillance (IEH/IOM, 2004; Searl, 2007). However, many different approaches are used for exposure measurements. These include differences in the use of personal and static monitoring, collection of different size fractions (respirable, inhalable, total), different analysis methods, (gravimetric, ICP, species) and differences in data metrics, storage, traceability and quality assurance procedures. Often, little contextual data is retained. These differences limit the utility of such data for exposure assessment and exposure reconstruction required for high quality epidemiology, particularly where a multi-centre approach is being developed.

Diverse approaches are also used to characterise the assessment of health end points in health surveillance programs.

The aims of this project were:

(i) to review methods for the evaluation of neurological health end points in the manganese industry and in other industries where these end points are considered relevant and to develop recommendations for a core set of evaluation methods to be used for the evaluation of these end points; and

(ii) to identify, develop and evaluate a set of methods, guidelines and tools to enable manganese producer companies to routinely collect valid, appropriate and comparable information relating to manganese exposure, applicable to current and future, as yet unplanned, epidemiological studies.

The first aim was completed during 2006 and a report is available upon request.

An extension to the project until 31 January 2008 was received in April 2007. Since then substantial progress has been made on the practical development of the exposure assessment tool and the guidance

However we have been unable to enlist the cooperation of sufficient companies to trial the tool and obtain feedback on the guidance. This has introduced further delays and the need to request a further extension to the contract until May 2008. During the period between now and May 2008, we would carry out a substantial trial of the tool at a site in Norway and possibly one other company. We will organise a workshop to disseminate information on the tools and guidance document to key stakeholders, and will try to work together with the IMnI to try to encourage participation of relevant stakeholders.

Body

The following describes the progress made on the second aim of the project since the start of the project

I) Neurological health end points

The review was concerned with identifying specific neurobehavioural tests which demonstrated any effects of occupational manganese exposure upon performance, and how reliable such effect-detection may be. A structured and systematic literature search was undertaken according to standard principles of evidence-based reviews. Sixteen databases were searched using pre-defined search terms and criteria, with the initial search identifying 153 articles, of which 49 were retained that reported on investigations of occupationally exposed workers or former workers, that involved neurobehavioural testing.

The pattern of results and methodologies used in the 49 studies were inconsistent and therefore inconclusive, although 12 studies were identified as being of better methodological quality. Given the variation in the range of outcome measures, uncertainties associated with (i) retrospective exposure measurement, (ii) the variety of testing apparatus, (iii) the variety in exposure scenarios and (iv) variability of testing conditions, this inconsistency was to be expected. Neurobehavioural investigations used in the field included a wide variety of batteries and a complex array of tests covering multiple functional domains, with most studies not explicitly stating the reasons for the choice of tests that were used.

In respect of the specific objectives of the current review, the following conclusions may be drawn. First, the literature did not provide convincing evidence of generally adverse effects of occupational manganese exposure upon neurobehavioural functioning. A lack of consistently demonstrable adverse effects among the better quality studies was strongly suggestive of this conclusion in terms of comparisons between exposed and non-exposed workers. Secondly, when the better quality studies were able to suggest the presence of effects in relation to different doses and levels of exposure, such effects were consistently detected by motor functioning tests of eye-hand coordination, tremor, and finger tapping (as well as reaction time tests to a lesser extent). However, such effects were difficult to explain in the absence of any reliable differences between exposed and non-exposed groups. It is therefore proposed that such dose-related effects have perhaps been additionally confounded by the inconsistencies and vagaries in methodological and procedural aspects of some neurobehavioural investigations.

Issues concerning the precise nature of neurobehavioural testing that may confirm such effects may therefore be difficult. It is worth noting that of the many tests used in neurobehavioural investigations, some of the better quality studies showed exposure effects that were limited to the domains of motor speed, coordination, memory, reaction time, and to a lesser extent cognition. It seems sensible to state that neurobehavioural tests concerning motor function, coordination and reaction time could be potentially useful when investigating performance differences between workers with varying levels of manganese exposure. However, such usefulness has not been demonstrated in investigations between exposed and unexposed workers. It should also be noted that no true longitudinal studies of neurobehavioural performance in both exposed and controls had been conducted, which would have allowed performances of specific tests to be compared over prolonged periods.

Future neurobehavioural testing in the area of occupational manganese exposure must include open and standard accounts of testing procedures, testing environments, testing personnel (and their related reliabilities), reasons for the choice of tests administered, and the reliability coefficients of the tests used. In addition, future neurobehavioural testing in the area of occupational manganese exposure should seek to use next generation tests, including “models of performance” such as Item Response Theory (IRT) and Computerized Adaptive Testing (CAT) that avoid reliability and measurement constraints suffered by contemporary neurobehavioural systems. As such tests are still currently in development and may be for some time, it is additionally recommended that future testing using current neurobehavioural methods should be restricted to the domains of motor functioning, coordination, memory, and reaction time. Specific tests which have yielded more “positive results” in the better quality studies included: “static-steadiness” and “tremor” as measured by accelerometers and tremometers (the Hole Test); “repetitive hand movements” as measured by the Movemap digital tablet, finger tapping, and orthokinesmeters. Tests of eye-hand coordination such as the Santa Ana Grooved Pegboard, the Movemap tablet, and the Pursuit Aiming test were also fruitful. Other tests which occasionally identified positive effects, albeit to a slightly lesser extent, included tests of simple reaction time and complex reaction time (although the specific tests were not identified in the older better quality studies). Tests of cognition and memory that also occasionally provided evidence of exposure effects included basic tests of memory (Digit Span) and learning (Digit Learning and Digit Symbol).

Due to the high variability existing between studies in terms of methodological quality, participant selection, tests used, recording of procedures, and exposure estimates, it seems unlikely therefore that neurobehavioral data will be able to make a contribution to the particular debate concerning manganese exposure in the near future unless more rigorous and open accounts of research methodologies and testing procedures are incorporated.

II) Exposure Assessment Tool

Task 1 Finalise the draft tools

Database design and development

A prototype exposure assessment database has been developed for the consistent collection, storage and analyses of manganese exposure data (working name ManganeX). This relational database is implemented in Microsoft Access, and has been developed to the point where a good working version can be demonstrated to and discussed with trialists. Besides identifying specific data items, with input and collaboration from industry

representatives we aim to incorporate the following features into the database, standardised as far as possible, across the Manganese industry.

- Adoption of EU & US standards for exposure data elements
- Common terms and definitions for manganese industry data items, parameters, characteristics and exposure survey and assessment data
- Common coding lists for shared data items
- Agreement on appropriate contextual data to included – to help describe & explain exposure
- Overall compatibility and uniformity in data formats – to facilitate the common methodology for data collection, storage and analysis, and potentially the transfer, amalgamation & sharing of (anonymised) data.

Other features of the database will include:

- User friendly interface design with menus and forms for data entry and management
- Data collection forms for exposure surveys
- Help & guidance materials
- Ability to produce reports and data summaries
- Output of exposure data for analysis by other applications

The data for manganese exposure assessments is arranged in three main related areas:

Company, with information on
- Company details; Employees; Jobs

Plant / Premises, covering
- Processes ; Workplaces; Tasks

Exposure surveys with details of
- Survey planning
  - Methods & strategy - sampling and analysis schemes;
  - Processes and workplaces surveyed
- Sample - collection and analysis results details
  - Sample type; jobs/operations; task; and additional contextual data
  - Analytical results; agents and concentrations

A generalised schematic of these is shown in figure 1 below.

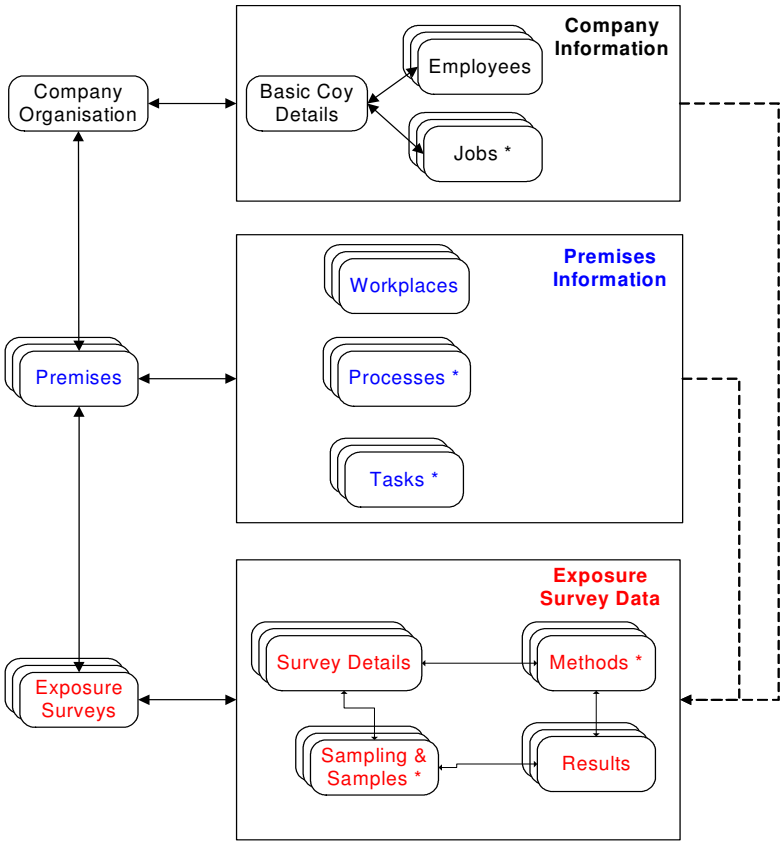

Figure 1: General schematic of data areas in Manganex

Data contents and definitions

Adapted from earlier IOM chemical exposure database work (Ritchie et al., 2004) a generalised conceptual data model of the database was produced to accommodate all of the data elements required, and the relationships between them. The overall conceptual model was subsequently further developed and refined using entity-relationship modelling techniques to design the relational database, and then its expression in Microsoft Access tables, and accessory lookup tables.

A key attribute of the database design is the ability to link related data both within, and between, the main areas. This allows links to be made for example between data at the company level (eg an employee and their current job), and data at the sample level (eg the job and tasks being done by the worker during sampling). In this way a bank of basic “reference” data about the company and premises is entered once, and referred (linked) to during sampling and exposure measurement, without necessarily having to repeat the data entry of the premises data.

A diagram of the database entity-relationship model in the current prototype implementation is shown below in figure 1. In order to better cater for the needs of industry and exposure assessment specialists the data model and consequently the database interface have been considerably redesigned and updated and since the previous progress report. Some aspects of these features are briefly described below.

A generalised entity-relationship diagram for the database is shown in figure 2. This is a simplified view of the more complex model within each of the three areas in Manganex and does not resolve sub-entities, many to many relationships, etc, at this point.

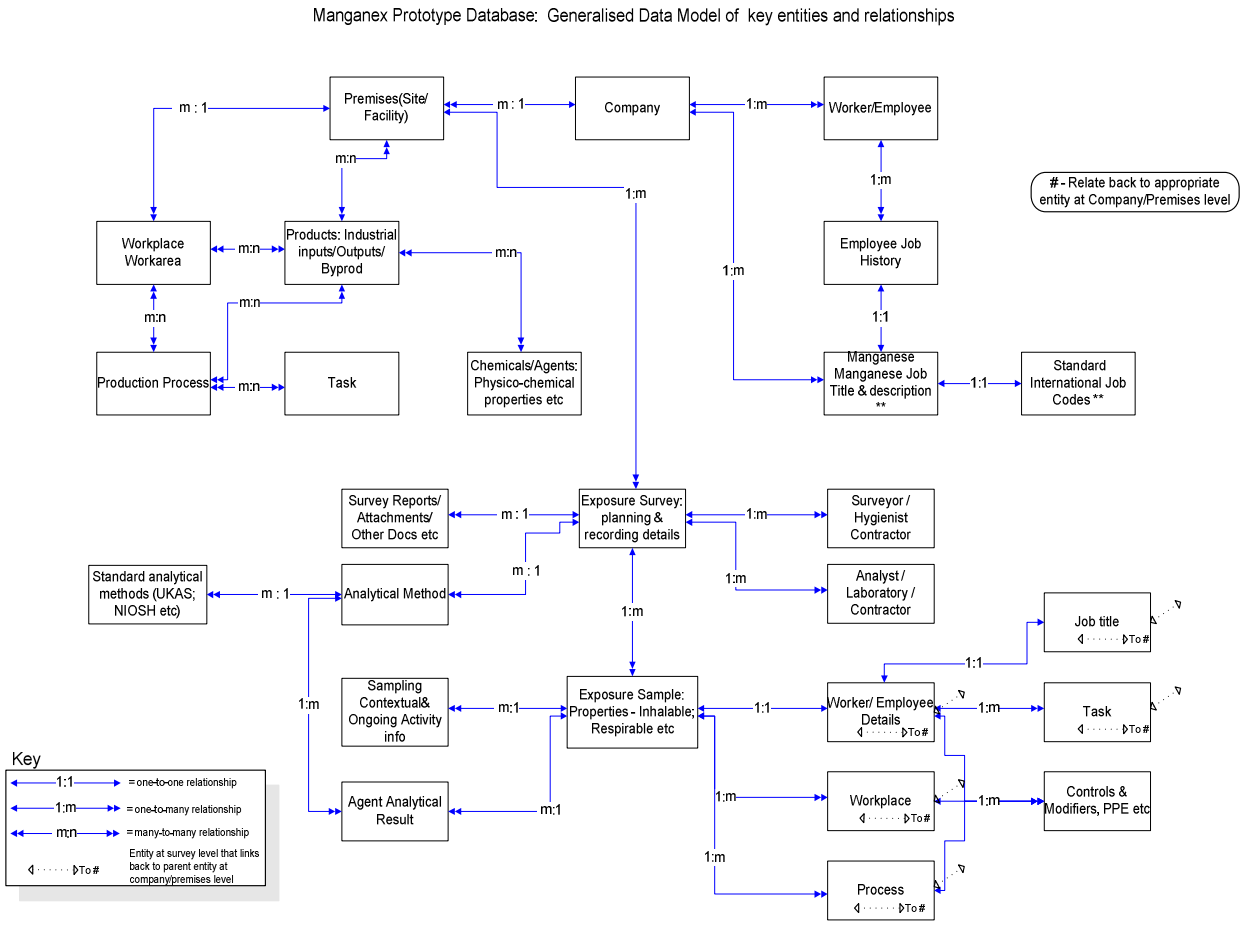

Figure 2: Overall entity relationship diagram for prototype Manganex database

The full data model is used to define a physical schema for the data base that define the actual tables used to implement the data entities. Coding or “lookup” tables are used to implement coding lists and drop down lists for the database interface.

As stated earlier, a principal objective is to create common or shared lists of codes for key entities across the manganese industry. The principal items identified and unified as far as possible to date are: Job, Process, Task, Generic Products/Materials, Clearly these will be augmented and supplemented as further contact with the industry as trials and other feedback continues. Similarly, classifications for other more general lookup data (eg survey type, analytical method and technique, sampling devices, and so on, are being encoded for industry wide use in the database. Currently there are xxx data tables and zzz lookup tables in the database. These will be more fully enumerated and documented in a subsequent report.

Interface development

In concert with development of the data model the functionality, layout and interface components of the database have likewise been substantially updated and improved. The following diagrams attempt to demonstrate the layout and look of the current prototype database.

The modules of the database that will manage: reports and summaries; data output/export; and the provision of help and guidance systems and materials, have only been addressed in a rudimentary manner to date. In the development process these features will be most efficiently and effectively addressed latterly, following the finalisation of the data contents, structures and user interface of the database.

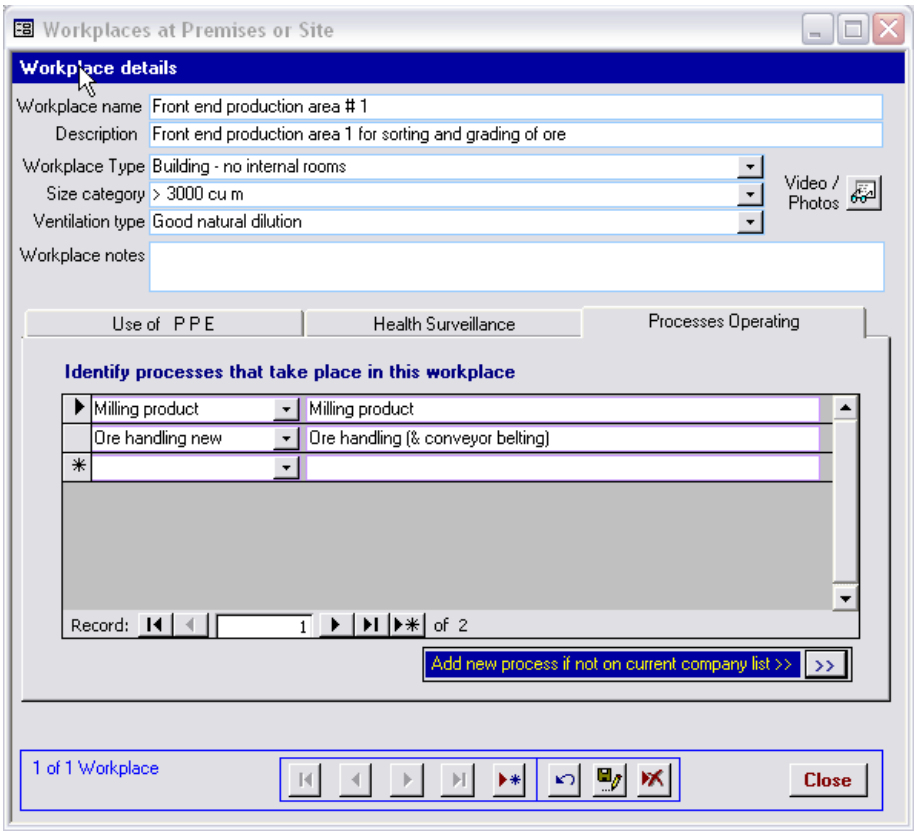

Figure 3: Interface demo - premises workplace level

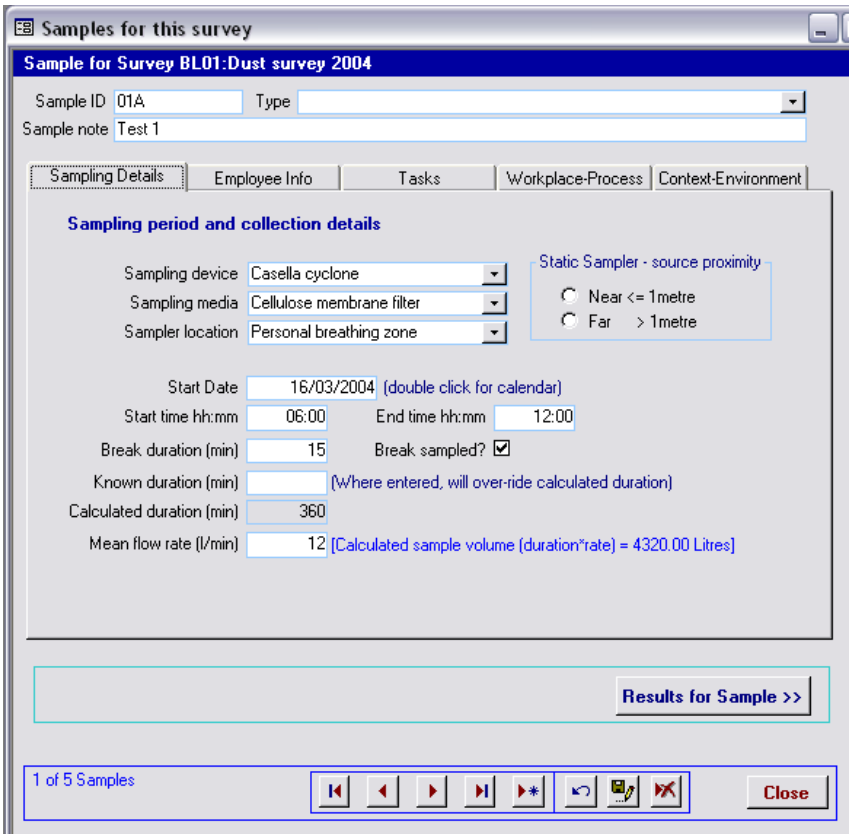

Figure 4: Interface demo – survey sample level

Exposure survey data collection forms

Based upon the data definitions to date, draft exposure data collection forms have been produced. These have been demonstrated to and are being reviewed by trialists. They will be subject to update following review and

as other data modifications to the database continue. It is intended that the forms will linked to a survey definition so that they can be output/printed for use in that survey with ID numbers and possibly other details pre-printed prior to actual data collection in the field. It is considered that beyond this project this methodology may be further developed to allow the collection of exposure data items into forms on handheld devices/PDAs used by field staff, for subsequent direct upload to the database, further reducing manual data entry.

Guidance document

A guidance document accompanies the database tool and describes sampling methods and strategies, need for contextual information, data entry procedures and advice on statistical analyses of the data. A draft of the guidance document is available upon request. Manganese exposure can be monitored with a variety of sampling and analytical methods, depending on the actual agent and the state of the contaminant. In addition, there will be regional differences in the preferred sampling method for airborne contaminants. For example, various sampling devices are available that collect the inhalable or respirable fraction of the airborne dust. It is well known that these devices do not always give comparable results. This guidance document does not prescribe standard methods for sampling manganese dusts and fumes, although a number of recommendations are made.

Little is known about dermal exposure to manganese and, to our knowledge, there are currently no results available from dermal exposure measurements in the manganese industry. Searl (2007) recommended that a pilot measurement campaign be carried out to determine the possible dermal exposure levels in this industry. Although we recognise the need for dermal monitoring, we have not considered dermal exposure here, but it is recommended that this be considered in the future.

The guidance document first describes issues to consider when developing a sampling strategy for a measurement survey, including a brief description of suitable sampling and analytical methods, quality control procedures and collection of contextual information. The guidance subsequently provides a brief overview of the database and instruction for use of the database, such as production of sampling forms and entering of exposure data and finally gives some guidance on statistical analyses of exposure data.

We have assumed that the user is familiar with the basics of occupational exposure monitoring. For a more detailed background on exposure monitoring and occupational hygiene, we refer to general occupational hygiene textbooks such as Occupational Hygiene by Gardiner and Harrington (2005).

Task 2 Planning/refining visits with companies

It was planned that the prototype tools (database and guidance) be taken to and discussed with at least two candidate manganese companies to elicit their views and input to the design, and that following these visits the prototype is be revised and finalised. We have visited one Mn producing company in Europe to demonstrate the database and guidance and receive general feedback to ensure that the database is aligned. Unfortunately we have so far had a poor response to requests to other European companies, but have sent reminders and we continue to press.

In the meantime however, following the first successful visit, the participating company is further commenting upon and updating coding and data collection forms. Also, many positive and constructive suggestions for features and refinements to the prototype made by the participants are currently being implemented in any case. One issue in particular that was brought up during this visit was the need for industry to collate exposure data for the purpose of risk assessment, and in particular REACH. We have subsequently, made some adjustments to the database, so that it is compatible with industry's requirement for the purpose of REACH, whilst retaining the its capacity to store data required for epidemiological studies.

Task 3 Field exercises

Following revision and refinement after the initial trials the prototype tools will be more extensively piloted in two field exercises with manganese companies. One company is positively willing to participate in the trials. The field trials are expected to take approximately 3 to 4 days, during which the exposure assessment tool will be trialled by company staff, with assistance from IOM staff. This will involve, the following tasks:

1) entering company level information;

2) entering survey details and producing sampling records;
3) collecting exposure measurements;
4) entering the measurement data; and
5) producing brief reports.

Following these exercises, final changes will be made to the database and the guidance prior to reporting. The companies that participated in the trials will receive a final version of the database.

Task 4 Presentation in a workshop
As originally envisaged, information about the tools and the outcomes of the field exercises will be disseminated to the wider manganese industry through a workshop to be held in Edinburgh in Spring (target May) of 2008.

Task 5 Final Report
The final report will include an electronic copy of the ManganeX database and guidance document.

Key Research Accomplishments

1) The health endpoints and methods to determine these have been reviewed and a report produced.
2) A prototype of the exposure database tool was developed and has been presented to one company manufacturing Mn.
3) A draft guidance document has been developed

Reportable Outcomes

Review of the methods and endpoints in studies relating to the neurobehavioural effects of occupational exposure to manganese was produced.

The final report of the project and any publications will be prepared after the extensive trial at a plant in Norway.

Conclusions
The review of the literature of neurological health end points in relation to Manganese exposure showed that a wide range of neurological health end points were used in the various studies. No consistent exposure response associations between Mn exposure and adverse neurological effects were observed, although some of the better quality studies adverse showed effects of manganese exposure on motor functioning tests of eye-hand coordination, tremor, and finger tapping, and to a lesser extent reaction time. It is essential that any future epidemiological study of neurological health end point in relation to Mn exposure use rigorous and open accounts of research methodologies and testing procedures are incorporated.

Future neurobehavioural testing in the area of occupational manganese exposure should concentrate on domains of motor functioning, coordination, memory, and reaction time. In addition, next generation tests, such as Item Response Theory (IRT) and Computerized Adaptive Testing (CAT) should be used (if and when these become available). Finally, all studies must include open and standard accounts of testing procedures, testing environments, testing personnel (and their related reliabilities), reasons for the choice of tests administered, and the reliability coefficients of the tests used.

Regarding the exposure assessment tool, we have received very positive feedback from one company in Europe but have struggled to enrol other companies in trials for the database. We believe that the database could be a very useful tool for the manganese industry for any future epidemiological studies as well as dealing with any regulatory requirements, such as REACH.

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TITLE: Cellular mechanisms involved in the uptake and delivery of inhaled manganese via the olfactory nerve

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14. ABSTRACT

Neurotoxicity is a significant public health concern associated with manganese (Mn) inhalation. Inhaled Mn is deposited in the olfactory epithelium and can be transported directly to the mammalian brain via the olfactory nerve. We hypothesize that the divalent metal transporter (DMT-1) plays a role in the initial uptake of inhaled Mn by the rat olfactory epithelium. We confirmed that DMT-1 has slightly higher expression in the rat olfactory epithelium when compared to the respiratory epithelium (1.5-fold, $p < 0.05$), developed viral delivery methods, pilot transfection studies using lentiviruses expressing green fluorescent protein (GFP) and Lac-Z. Since the inception of this project other investigators have confirmed the role of DMT-1 in the olfactory transport of manganese.

15. SUBJECT TERMS
Manganese, neurotoxicology, olfactory transport, brain delivery

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Introduction

Neurotoxicity is a significant public health concern linked with high-dose manganese inhalation. People at risk for manganese neurotoxicity include welders and other workers involved in metal smelting operations, steel production, and foundries. A critical step in the pathogenesis of manganese neurotoxicity is the initial accumulation of the metal in the brain. Unlike many xenobiotics, inhaled manganese deposited in the olfactory epithelium can be transported directly to the brain via the olfactory nerve. Direct nose-to-brain (i.e., olfactory) delivery of manganese has been observed in multiple animal species, including nonhuman primates, suggesting that this route of delivery could occur in people. Few studies have evaluated the mechanism by which manganese is absorbed by the olfactory epithelium and then ultimately transported. We hypothesize that the divalent metal transporter (DMT-1) plays a role in the initial uptake of inhaled manganese by the rat olfactory epithelium. We have chosen the rat because the olfactory transport of manganese has been well studied in this species.

Manganese neurotoxicity is a significant public health concern associated with welding, metal smelting operations, steel production, and foundries. Under conditions of high occupational exposure, excess manganese accumulates within the human striatum and globus pallidus and produces damage to dopaminergic neurons within these sites (Perl and Olanow, 2007). A critical step in understanding whether inhaled manganese plays a role in chronic neurological disease is to determine exposure conditions that lead to increased concentrations of the metal within the central nervous system. This understanding is especially critical for manganese since its mechanism of toxicity is poorly understood and since an elevation in brain manganese levels is one of the few reliable biomarkers available to monitor for excessive exposure (Andersen et al., 1999). Brain delivery of manganese is higher following inhalation versus ingestion, and pharmacokinetic factors that may contribute to this increased efficiency in brain manganese delivery include increased manganese absorption from the pulmonary tract, slower blood clearance of absorbed manganese, and direct delivery to the brain via the olfactory system (Aschner M, Dorman DC, 2006).

Olfactory transport of manganese has been demonstrated to occur in the rat, mouse, and freshwater pike following intranasal instillation (Gianutsos et al., 1997; Tjälve and Henriksson, 1999; Tjälve et al., 1995, 1996) or inhalation (Brenneman et al., 2000; Dorman et al., 2002; and Elder et al., 2006). Collectively, these findings suggest that the olfactory route may indeed be a significant pathway by which inhaled manganese gains direct access to certain structures within the rat brain. Cellular mechanisms associated with the initial uptake of manganese by the olfactory epithelium, translocation of manganese within the olfactory neuron, and subsequent transport of manganese by the olfactory nerve are incompletely understood. The divalent metal transporter 1 (DMT-1) is involved in the intestinal uptake of numerous divalent metal cations and animals with a defective DMT-1 allele (i.e., homozygous Belgrade (b/b) rats) have reduced uptake of manganese to the central nervous system (Chua and Morgan, 1997).

To date various animal models have been used to investigate mechanisms by which manganese is absorbed by epithelial membranes, transported in the blood, or delivered to more distant tissues. The application of RNA interference (RNAi) provides an attractive alternative for the study of metal transporters. This technology allows the mRNA of the gene of interest to be specifically degraded inside the cell by the addition of complementary 21 to 25 nucleotide dsRNA molecules (called small inhibitory RNAs or siRNAs) leading to a virtual “knock-out” of the gene (for review see Hannon, 2002). Delivery of the siRNAs can be achieved through several different vehicles. In cells or tissues that can be easily transfected, synthetic siRNA duplexes can be directly transfected using lipid and non-lipid based methods. For non-transfectable primary cells, an effective alternative to synthetic siRNAs is the in vivo expression of short hairpin RNAs (shRNAs) following viral delivery. The shRNAs consist of two short inverted repeats separated by a hairpin loop sequence and expressed using a Pol III or modified Pol II promoter. The resulting RNA molecule forms an intramolecular stem-loop structure inside the cell and is processed enzymatically into a functional siRNA duplex. Expression of shRNAs leads to target gene silencing and allows experimental approaches similar to those used in knockout animals to explore gene function

Our original hypothesis was that DMT-1 plays a role in the initial uptake of inhaled manganese by the rat olfactory epithelium. We expected to develop adenoviral and lentiviral shRNA vectors for DMT-1 (objective 1), perform intransasal delivery of these vectors with subsequent demonstration of reduced DMT-1 mRNA and/or

protein (objective 2), and subsequently confirm that olfactory transport of manganese (as ^{54}Mn) is decreased on the side of the head with degraded nasal (olfactory epithelial) DMT-1 mRNA (objective 3).

Previous progress reports described efforts to develop the appropriate adenoviral and lentiviral shRNA vectors for DMT-1 and pilot transfection studies. As noted in these progress reports two ongoing problems were encountered. First, auto-fluorescence present within the rat olfactory epithelium interfered with our ability to detect the presence of a green fluorescent protein (GFP) based reporter system following viral transduction. Second, and more importantly, transfection rates associated with the viruses were consistently lower than anticipated based on work published by other investigators using mouse models and prevented sufficient delivery of the viral vectors that contained shRNA vectors for DMT-1.

During the course of these studies, other investigators (Thompson et al., 2007) demonstrated that Belgrade rats, an animal model with a glycine-to-arginine substitution (G185R) in their DMT1 gene product that alters iron and manganese metabolism, have reduced absorption of intranasally instilled ^{54}Mn. Thompson and coworkers also provided immunohistochemical evidence that DMT1 was localized to both the lumen microvilli and sustentacular cells found within the olfactory epithelium. These data provide strong evidence that DMT1 does indeed play a role in olfactory manganese absorption thereby confirming our original hypothesis.

Body

Olfactory epithelial DMT-1 expression. Naïve 10-week old male and female CD rats were deeply anesthetized with sodium pentobarbital (60 mg/kg) and exsanguinated. Nasal samples, consisting of the nasal septum, and the naso-, maxillo-, and ethmoid turbinates, were dissected free from the nasal cavity. The nasal septum, and naso- and maxillo- turbinates were used as a source for respiratory epithelium and the ethmoid turbinates were used as source for olfactory epithelial cells. The respiratory and olfactory epithelium was manually dissected directly from the boney and cartilaginous nasal turbinates and septum. The absence of epithelium from turbinate and septal tissues was confirmed via histology. Nasal respiratory and olfactory epithelial samples were homogenized in TRIZOL® Reagent, 1 ml /50-100 mg of tissue, and separated by centrifugation in 0.2 ml of chloroform /1 ml of lysis reagent using heavy Phase Lock Gel™ tubes. RNA was precipitated from the aqueous phase using isopropyl alcohol and pelleted with centrifugation. The pellet was washed and applied to the QIAGEN RNeasy Mini kit column. RNA was digested free of DNase on-column using the QIAGEN RNase-Free DNase Set, washed in buffer, and eluted with RNase-free water. Quality of the RNA was verified with standard spectrophotometric analysis and gel electrophoresis using the Agilent 2100 Bioanalyzer. Preparation of the probe and hybridization to the microarray was performed by CIIT's Gene Expression Core. Double-stranded cDNA was synthesized from RNA samples using an oligo-dT24-T7 from 5 µg of total RNA/sample. Synthesized cDNA template was transcribed to biotin-labeled cRNA using the GeneChip® IVT Labeling Kit. Fifteen µg of labeled cRNA was fragmented and hybridized to Affymetrix Rat Genome 430 2.0 arrays in the Hybridization Oven 640 for 16 hours at 45°C. After hybridization, arrays were washed using the GeneChip® Fluidics Station 450 and scanned with the GeneChip® Scanner 3000.

Expression data were preprocessed using RMA with a log base 2 (log2) transformation. Statistical analysis of the microarray data was performed in R using the affylmGUI package. To identify genes with significant changes in expression between tissue types and gender, data were analyzed using a linear model with contrasts between male and female and respiratory and olfactory epithelium. Probability values were adjusted for multiple comparisons using a false discovery rate of 1% (FDR = 0.01). Genes identified as statistically significant were subject to an additional filter by selecting only those genes that exhibited a $\geq$ 2-fold change. Analysis of gene ontology (GO) categories was performed using Onto-Express. Affymetrix probe set identifiers for the genes of interest were uploaded to the Onto-Express web site (http://vortex.cs.wayne.edu/index.htm) and analyzed based on the Affymetrix U133A_2 reference list. A hypergeometric test was performed to identify GO categories with significantly enriched gene numbers with *p*-values corrected using a false discovery rate of 5% (FDR = 0.05). The resulting list of GO categories was further refined by removing categories containing > 4 genes.

We confirmed DMT1 mRNA expression in the rat nasal epithelium (Roberts et al., 2007). DMT-1 had slightly higher expression in the rat olfactory epithelium when compared to the respiratory epithelium (1.5-fold, $p < 0.05$). The project will develop quantitative PCR and protein analysis methods (Western blots) for DMT-1 in the olfactory epithelium from naïve and viral vector-treated rats.

Vector production: Prior to performing shRNA knockdown of DMT1, we first assessed the tropism and infectivity of adenovirus and lentivirus in the nasal epithelium using a constitutively expressed LacZ reporter. Adenovirus containing a LacZ reporter was purchased from a commercial source in a preconcentrated and purified form (Clontech, Mountain View, CA). Lentivirus containing a LacZ reporter was generated internally using a commercial lentiviral expression vector (Invitrogen, Carlsbad, CA). Briefly, the lentiviral expression vector was cotransfected into HEK-293T cells with several packaging plasmids that supply helper functions as well as envelope and replication proteins based on descriptions by Blömer and coworkers (1997). The resulting virions were harvested from the media and concentrated by centrifugation at 50,000 g for 1.5 hrs. The concentrated virus was titered using Taqman primer/probe chemistry specific for the RRE region of the lentivirus (Lizee et al., 2003). Several production runs were completed for the Lenti-LacZ vector. Estimated virus concentrations initially were low (1.3×10^7 to 2×10^7 virions/ml) and recovered volumes were also low (250 µl). Subsequent production efforts yielded significantly higher amounts (> 500 µl) of more concentrated (1×10^{11} virions/ml) viral particles. A lentivirus vector (pTK113) containing a green fluorescent protein (GFP) reporter was similarly generated at CIIT. Virus concentrations for the Lenti-GFP vector ranged from 1.9×10^7 to 8.9×10^7 virions/ml, with production volumes of 10 to 250 µl.

Using the BLOCK-iT™Lentiviral Pol II miR RNAi expression system, each of the three siRNA oligos were inserted into an entry clone containing attL sites using a BP combination reaction. The entry clone was then used in an LR recombination reaction with pLenti6/V5-DEST vector to create a lentiviral expression clone. The lentiviral expression vector of each siRNA was then co-transfected into HEK-293T cells with several packaging plasmids that supply helper functions as well as envelope and replication proteins. The resulting virions were harvested from the media and concentrated by centrifugation at 50,000 g for 1.5 hours. The concentrated virus was titered using Taqman primer/probe chemistry specific for the RRE region of the lentivirus (Lizee et al, 2003).

Pilot studies examining the use of an Adeno-LacZ viral vector: Initial efforts focused on administration of the Adeno-LacZ vector. Methods used in this pilot study were modified from Ivic et al. (2000). Virus particles in 2.5% glycerol in 0.1% Hank's Buffered salt solution were slowly infused (10-100 µl volume delivered over 10-60 minutes; 1×10^{10} ifu/ml) into the right nostril of three ketamine/xylazine anesthetized rats. Two days after infusion, rats were anesthetized with ketamine and xylazine and perfused intracardially with cold 4% paraformaldehyde and 0.2% glutaraldehyde. The heads were spilt along the midline and stained by enzyme histochemistry for LacZ using the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Following staining with X-gal, the tissues were placed in 70% ethanol and processed for paraffin embedding. Coronal 3-5 µm sections at six standard locations as described in Méry, et al. (1994) were counterstained with eosin. Non-specific staining was present on both sides of the nose, primarily in the respiratory epithelium, and could be seen *in situ* as well as in sectioned tissue. No LacZ staining was seen in larynx or lungs from these animals.

Pilot studies examining the use of a Lenti-GFP viral vector: Lenti-GFP vector was produced at CIIT and administered to three rats. Virus particles in 2.5% glycerol in 0.1% Hank's Buffered salt solution were infused (30 or 100 µl volume delivered over 30-40 minutes; ~8.9×10^7 ifu/ml) into the right nostril of two ketamine/xylazine anesthetized rats. Three or five days after infusion, rats were anesthetized with ketamine and xylazine and perfused intracardially with cold 4% paraformaldehyde and 0.2% glutaraldehyde. Whole turbinates and the nasal septum were dissected and observed using confocal microscopy (Zeiss LSM 510). GFP was visualized directly with filters set to 485 nm for excitation and 520 nm for the emission wavelengths. The turbinates and septum were then paraffin-embedded and sectioned and stained by immunohistochemistry (IHC) for GFP. A smaller volume (5 µl) of the Lenti-GFP vector was drip instilled into several additional rats. Animals were euthanized by carbon dioxide asphyxiation four days later, then ethmoid turbinates were collected and cultured overnight. The explants were observed by confocal microscopy immediately after collection and 24 hours later. Unexpectedly, high levels of auto-fluorescence were detected in the nasal tissues from both instilled and control sides of the nose in all three

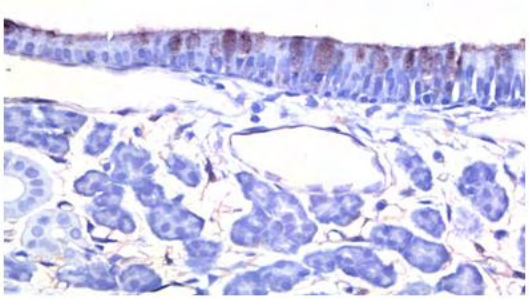

Figure 1. Goblet cells in rat nasal respiratory epithelium stained positive for GFP using anti-fluorescein antibody. Tissues were collected 3 days after the rat was infused with 100 µl of Lenti-GFP vector.

animals. Autofluorescence negated our ability to determine whether transfection occurred. Immunohistochemistry (IHC) of processed nasal tissues collected 3 days post-instillation using anti-fluorescein rabbit polyclonal IgG Fab fragment (Molecular Probes, A-6413) also revealed non-specific and high background staining that was predominantly limited to the goblet cells.

Pilot studies examining the use of a Lenti-LacZ viral vector: Lenti-Lac Z viral vectors were produced as described above. One rat was anesthetized with ketamine/xylazine and infused with 100 µl of Lenti-LacZ virus (1 X 10^{10} ifu/ml) for 50 minutes. It was anesthetized and perfused with cold 4% paraformaldehyde and 0.2% glutaraldehyde five days after viral infusion. Nasal turbinates, septum and olfactory bulb were collected and stained by enzyme histochemistry for LacZ., then tissues were paraffin-embedded, sectioned at 3-5 µm, and counter-stained with eosin. Positive staining for LacZ was seen in patches on the septum and ethmoid turbinates 5 and 6 of the infused side, but not on the non-infused side. Minimal staining was present on the respiratory epithelium. Six additional anesthetized rats were given 50 µl of Lenti-LacZ (1 X10^{10} ifu/ml) by drip instillation over 5-10 minutes. They were killed by carbon dioxide asphyxiation and exsanguination five days later and the noses were processed for formalin-fixed paraffin embedded sections. Standard nasal sections were collected as described by Méry, et al. (1994) for IHC using rabbit polyclonal anti-beta galactosidase (Abcam, ab616) and SuperPicTure polymer detection kit (Zymed, with broad-spectrum AEC, 87-9963). Immunohistochemical staining of these sections confirmed transfection.

***In vitro* olfactory epithelial explant cultures**: We developed an alternative *in vitro* olfactory epithelial collected from male CD rats obtained from Charles River Laboratories, Inc. (Raleigh, NC) (Roberts et al., 2006). Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg) and exsanguinated by severing the abdominal aorta. Immediately after death, the head with the lower jaw and skin removed was split in half along the medial longitudinal suture. The proximal nasal septum (between the anterior surface of the incisor teeth and the incisive papilla) and ethmoid turbinates were removed by careful blunt dissection using fine ophthalmic surgical instruments as described in Uraih and Maronpot (1990). The ethmoid turbinates and nasal septum are used as the source of the olfactory epithelial explants (Figure 2). The ethmoid turbinates and septum were separated and cut into small pieces. Individual sections were placed onto tissue culture-treated Transwell mesh inserts (6-well plates with clear 0.4 µm pore-size inserts; Corning Costar, Cambridge, MA). Incubation media (Clonetics' KGM BulletKit, Cambrex Corporation, East Rutherford, NJ) is added to the basal (2 mL) and apical (0.5 mL) chambers. Phenotypically normal appearance can be maintained for approximately 36-48 hr with this explant system. This duration of explant viability was suitable for our planned uptake studies.

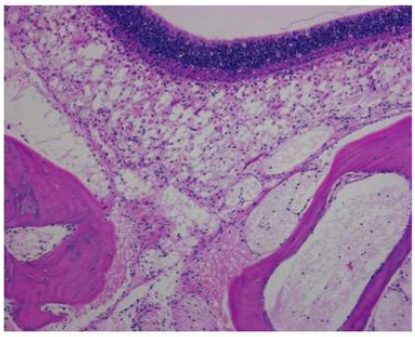

Figure 2. Photomicrograph of a rat olfactory epithelial explant culture showing the intact epithelium and associated nerve fibers.

One set of explants was treated with 0 to 1000 µl of Lenti-GFP virus in the media for four hours. The media was replaced and the explants were viewed using confocal microscopy immediately post-incubation and after 24 hours. Tissue autofluorescence was observed in control and viral-treated explants at the initial and 24-hour time points. No specific staining could be seen related to the viral treatment. This duration may be too short for the production of GFP within the cells; however, the explants are not viable for more than 48 hours. Another set of explants was exposed overnight to varying amounts of Lenti-LacZ virus, and then stained 24 hours later for LacZ by enzyme histochemistry. Surprisingly, positive staining for beta galactosidase was observed in treated as well as media-exposed control explants. Increased beta galactosidase activity is associated with cell death and apoptosis (Gerland, et al., 2004) and senescence (Itahana et al., 2007).

Key Research Accomplishments

- Confirmation of DMT1 mRNA expression in the rat nasal epithelium.
- Development of lentiviral vectors with green fluorescent protein (GFP) and beta galactosidase (LACZ) expression markers.
- Further developed nasal explant systems for *in vitro* virus transfection studies. These methods allow method development efforts to rely on the use of fewer animals and takes advantage of tissue samples from naïve rats that would otherwise be discarded.
- Developed viral vector containing short hairpin RNA for DMT-1.

Reportable Outcomes:

Roberts ES, Soucy NV, Bonner AM, Page TJ, Thomas RS, Dorman DC (2007). Basal gene expression in male and female Sprague-Dawley rat nasal respiratory and olfactory epithelium. *Inhal Toxicol*. 19:941-949.

Individuals receiving support from the grant:

- David Dorman, DVM, PhD (< 3%)
- Rusty Thomas, PhD (<3%)
- Anna Bonner, BA (< 3%)
    - Ms. Bonner left the institute April 3, 2006
- Kelly Miner, BS (< 3%)
    - Ms. Kelly Miner was assigned to this project in April, 2006; however she left CIIT on 5/26/06
- Trista Kohel, BS (< 1%)
    - Ms. Kohel joined CIIT mid- summer and worked for several months in our laboratory learning a variety of animal handling and technical skills. She has since accepted a transfer to our analytical chemistry unit.
- Linda Pluta, BS (< 3%)

Conclusions

Work associated with Specific Aim 1 was completed. Work completed to date has confirmed the presence of DMT1 mRNA in the rat nasal epithelium. Our transfection studies with GFP or LacZ reporter systems have been hampered by unexpected autofluorescence of the rat nasal epithelium, low yield production runs for the viral vectors, staff availability, and most importantly low transfection rates with our viral vectors.

A no-cost extension of the project was provided in 2007; however in light of published work demonstrating the role of DMT1 in the olfactory transport of manganese we have not pursued completion of the remaining specific aims this year. Remaining funds associated with this project will be returned to Vanderbilt University.

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MHRP

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PRINCIPAL INVESTIGATOR: James Antonini PhD, Diane Miller PhD, James O'Callaghan PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203

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14. ABSTRACT

Questions persist regarding a possible association between neurological effects in welders and the presence of manganese in welding fume. Researchers have suggested that welding is not only a high-risk occupation for the development of manganism, but it may also be a risk factor for or can accelerate the onset of idiopathic Parkinson's disease. However, toxicology studies investigating this issue are lacking. The objective was to examine the potential neurotoxic effect of manganese in rats after pulmonary exposure to different welding fumes. Manganese was found to translocate from the lungs via the circulation to striatum and from the nasal airways to the olfactory bulb after inhalation in short-term studies. Consistent with the observed accumulation of manganese in the brain, intratracheal instillation of welding fumes differentially elicited neuroinflammatory responses in the olfactory bulb, striatum, and midbrain. Subchronic 90-day inhalation exposure studies in animals are ongoing to make more definite conclusions about the potential neurotoxicity of welding fume.

15. SUBJECT TERMS
Manganese, welding, neurotoxicity, inhalation

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Introduction

Epidemiology suggest that inhalation of welding fumes may cause adverse health effects in exposed workers. However, more information is required to determine causality, to evaluate temporal and dose-response relationships, and to elucidate mechanisms. To accomplish this, it is necessary to develop a welding fume generation and animal inhalation exposure system to perform long-term toxicology studies. The Health Effects Laboratory Division within CDC-NIOSH at Morgantown, WV has constructed a completely automated, robotic welding fume inhalation system that can expose laboratory animals to tightly-controlled, well-characterized welding fumes generated from different welding processes and materials.

Serious questions have been raised regarding a possible causal association between neurological effects in welders and the presence of manganese in welding consumables. Some researchers have suggested that welding is not only a high-risk occupation for the development of manganism, but that it may also be a risk factor for or can accelerate the onset of idiopathic Parkinson's disease. However, toxicology studies currently investigating this issue are greatly lacking.

The objective of the study was to examine the potential neurotoxic effect of manganese in rats after pulmonary exposure to different welding fumes. Rats were exposed by inhalation or intratracheal instillation to welding fumes that contained differing levels of manganese. The translocation of deposited metals from the respiratory tract to other organs systems, including the central nervous system, was determined. In addition, integrity of the blood brain barrier (BBB), neuroinflammation, and oxidative/cellular stress responses were assessed to evaluate and define adverse neurotoxic effects.

Body

STUDY 1- Intratracheal Instillation of Welding Fumes Containing Different Levels of Manganese

The objective was to compare the neurotoxicity and translocation of metals from the respiratory tract to specific brain regions and other organ systems after intratracheal instillation of a welding fume that is high in manganese content compared to one that is lower in manganese content. Male Sprague-Dawley rats were treated with gas metal arc-mild steel (GMA-MS) welding fume or manual metal arc-hardsurfacing (MMA-HS) welding fume. These welding fumes were chosen on the basis of their varying metal composition, as well as, differences in their solubility, factors that could influence translocation. The GMA-MS fume was composed of iron (~85 %) and manganese (~15 %) and was mostly insoluble in water with a soluble/insoluble ratio of 0.014. The MMA-HS fume was higher in manganese content (~51 %) with lower levels of iron (~20 %) and found be more water soluble with a soluble/insoluble ratio of 0.218. The rats were treated by intratracheal instillation with 0.5 mg/rat of the GMA-MS or MMA-HS fumes once a week for 7 or 11 weeks. Control animals received intratracheal instillations of saline vehicle.

Exposure to either GMA-MS (low Mn) or MMA-HS (high Mn) resulted in distribution of manganese to extrapulmonary targets. Significant accumulation of manganese in the striatum, a brain area associated with dopaminergic control of motor function, was observed for both fumes following 7 weeks of exposure. Interestingly, the increases in manganese levels observed in the striatum at 7 weeks were not observed after 11 weeks of welding fume treatment. Following 11 weeks of exposure, significant increases in manganese concentrations were observed in the blood, lungs, hearts, and kidneys of the animals treated with the MMA-HS welding fume compared to the GMA-MS fume. These findings suggest that manganese can

potentially translocate from lung to different organ systems and may possibly elicit adverse systemic effects. Exposure to the MMA-HS fume caused a significant increase in pulmonary injury and neutrophil influx into the lungs compared to the GMA-MS fume and saline control groups. Slight, but not significant difference in lung responses was observed when comparing the GMA-MS and saline groups.

Neuronal damage and the subsequent cell death that occurs following neurotoxic insults can be mediated by several mechanisms that include inflammation, mitochondrial dysfunction, oxidative damage and excitotoxicity. Microglia, the macrophages of the brain play a crucial role in brain inflammatory responses. Pro-inflammatory cytokines and chemokines elicited by microglia can initiate mitochondrial impairment and oxidative stress; therefore, an inflammatory response may serve as the basis for neurotoxicity.

Consistent with the observed accumulation of manganese in the brain, intratracheal instillation of MMA-HS or GMA-MS welding fumes for 7 or 11 weeks differentially elicited neuroinflammatory responses in the olfactory bulb, striatum, and midbrain, brain areas that are predominantly dopaminergic; the latter two being commonly affected in PD. Significant increases were observed for TNF-α and CCL2, two pro-inflammatory cytokines, after treatment with the GMA-MS and MMA-HS fumes. An interesting observation was that the neuroinflammatory response in the olfactory bulb preceded (observed following 7 weeks of exposure) the response seen in striatum or midbrain (seen only following 11 weeks of exposure). These findings suggest that early changes in the olfactory bulb may indicate a potential progression of neurodegeneration in other dopaminergic areas. Analysis of olfactory responses may therefore serve as an early indicator of a neurotoxic insult, particularly to the dopaminergic pathway.

Whether the neuroinflammatory changes observed are a direct consequence of the translocation of manganese to the brain, or an indirect systemic effect mediated by circulating cytokines and chemokines are yet to be determined. Regardless of how welding fume-mediated neuroinflammation in the brain occurred, the temporal induction CCL2 and TNF-α may suffice to elicit oxidative burst and trigger microglial activation. The actions of such proinflammatory mediators can contribute to manifestation of neurological, neurodegenerative or neurobehavioral conditions.

Neuronal injury is often associated with cerebrovascular dysfunction and modification of BBB function. Systemic inflammatory and oxidative stress events can disrupt BBB and allow translocation of toxicants to the brain. Following pulmonary exposure to each welding fume, we found increased expression of endothelial cell marker, ICAM-1 and extracellular matrix marker, MMP-9 in the striatum and midbrain at 11 weeks. These findings likely suggest a compromise of BBB function and remodeling. Convincingly, the regional expression of MMP-9 and ICAM-1 was consistent with the region-selective neuroinflammatory changes observed, indicating that areas that had compromised BBB permeability were likely susceptible to welding fume-induced neural toxicity.

STUDY 2- Inhalation of gas metal arc-mild steel welding fume

To evaluate temporal and dose-response relationships and to elucidate the mechanisms associated with the health effects of welding, a welding fume generation and animal inhalation exposure system is needed to perform long-term toxicology studies. A completely automated, robotic welding fume inhalation system that exposes laboratory animals to tightly-controlled, well-characterized welding fumes generated from different welding processes and materials has been developed. The physical and chemical composition of welding fumes and gases generated by the system have been characterized and found to be comparable to what is observed in the workplace.

Male Sprague-Dawley rats were exposed to 40 mg/m^3 of GMA-MS welding fume for 3 hours/day for 3 or 10 days. Longer-term exposures to GMA-MS fume for 30 and 90 days and to a fume with greater manganese content also are planned as part of the study but have not been completed at this time. GMA-MS was initially chosen for study in the initial experiments because a large majority of welders in the U.S. (~90 %) are exposed to this particular fume. In the characterization of the generated fume, the majority of the collected particles were observed in the fine size range with cut-off diameters of 0.10-1.0 □m. Additional nanometer-sized particles in the range of 0.010-0.10 um as well as larger, coarse particles with diameters >1.0 um in size also were collected. The mass median aerodynamic diameter was calculated to be approximately 0.31 □m. Electron microscopic analysis demonstrated that most of the aerosols generated were arranged in homogeneous, chain-like agglomerates of nanometer-sized primary particles. Metal analysis indicated that the particles were composed primarily of iron (80.6 %) and manganese (14.7 %).

The presence of iron and manganese was measured in different organs after exposure to assess the translocation of metals from the respiratory system. Significant elevations in both iron and manganese were observed in lungs after 10 days of exposure to GMA-MS welding fume compared to air control. A slight, but not significant, increase in manganese was measured in whole blood of animals exposed to the GMA-MS welding fume. A significant increase in iron was observed for the welding fume group in liver compared to air control. In addition, a significant elevation in manganese was observed in the kidney after exposure to GMA-MS welding fume. Importantly, no significant increases in iron and manganese accumulation were observed in any of the specific brain regions, except for the olfactory bulb. At 1 hour after the final exposure of the 3-day exposure regimen, a significant increase in manganese was measured in the olfactory bulb after inhalation of GMA-MS fume. However, by 24 hours after the last exposure, the manganese levels in the olfactory bulb returned to control levels, indicating a rapid rate of clearance of the manganese.

In examining lung responses at different time points after exposure for 10 days, no significant differences were observed in any of the lung injury or inflammation parameters when comparing the GMA-MS welding fume group with the air control group. In addition, no evidence of biochemical cellular damage or pathological changes was observed in the different brain regions after short-term GMA-MS exposure. The measurement of mediators of neuroinflammation, oxidative stress, and breakdown of the BBB in the samples collected from the exposed animals is ongoing. There is a need to extend the welding exposures for longer periods of time (e.g, subchronic exposures for 90 days). Whether the neuroinflammatory changes observed result in long-term neurotoxicity or are a direct consequence of the translocation of manganese to the brain, or an indirect systemic effect mediated by circulating cytokines and chemokines are yet to be determined.

Key Research Accomplishments

-A welding fume generation and inhalation exposure system was developed to expose laboratory animals.

-The generated welding fume was comparable to fume generated in the workplace after determining size distribution using a Moudi particle sizer and metal composition using inductively plasma coupled-atomic emission spectroscopy (ICP-AES).

-Animals were exposed to 40 mg/m^3 of gas metal-mild steel welding fume (the most common welding fume) for 3-hours/day for 3 and 10 days. Longer-term exposure for 30 and 90 days and exposure to fumes with different manganese content are ongoing.

-A separate set of animals were treated by intratracheal instillation of 0.5 mg/rat of gas metal arc-mild steel fume or a high manganese hardsurfacing welding fume once a week for 7 or 11 weeks.

-Specific brain regions and different organs were harvested from all exposed animals for histopathology analysis and measurement of metal content (e.g, Mn, Fe) by ICP-AES.

-Lung inflammation and injury were assessed after bronchoalveolar lavage analysis in all exposed animals.

- Integrity of the blood brain barrier (BBB), neuroinflammation, and oxidative/cellular stress responses were assessed to evaluate and define adverse neurotoxic effects.

Reportable Outcomes

1. Manuscripts

Antonini JM, Afshari AA, Stone S, Chen B, Schwegler-Berry D, Fletcher WG, Goldsmith WT, Vandestouwe KH, McKinney W, Castranova V, and Frazer DG. Design, Construction, and Characterization of a Novel Robotic Welding Fume Generation and Inhalation Exposure System for Laboratory Animals. J Occup Environ Hyg 3:194-203, 2006.

Antonini JM, Santamaria A, Jenkins NT, Albini E, and Lucchini R. Fate of manganese associated with the inhalation of welding fumes: Potential neurological effects. Neurotoxicol 27:304-310, 2006.

Antonini JM, O'Callaghan JP, Miller DB. Development of an animal model to study the potential neurotoxic effects associated with welding fume inhalation. Neurotoxicol 27:745-751, 2006.

Antonini JM, Sriram K, Benkovic SA, Stone S, Roberts JR, Frazer DG, O'Callaghan JP, and Miller DB. Effect of mild steel welding fume after short-term inhalation exposure in rats: Tissue metal deposition and neurological responses, in preparation.

Antonini JM, Roberts JR, Benkovic SA, Sriram K, O'Callaghan JP, and Miller DB. Neurotoxic responses in rats after intratracheal instillation of welding fume with varying concentrations of manganese, in preparation.

2. Abstracts

Antonini JM, Miller DB, and O'Callaghan JP. Characterization of welding fumes and their neurotoxic effects. 22nd International Neurotoxicology Conference: Manganese Symposium, Research Triangle Park, NC, September 2005.

Antonini JM, O'Callaghan JP, and Miller DB. Characterization of welding fumes and their potential neurotoxic effects. International Workshop: Neurotoxic Metals- Lead, Mercury, and Manganese, From Research to Prevention. Brescia, Italy, June 2006.

Antonini JM, Roberts JR, Benkovic SA, Sriram K, O'Callaghan JP, and Miller DB. Potential neurotoxic responses in rats after pulmonary administration of welding fume with varying concentrations of manganese. 23rd International Neurotoxicology Conference: Health Effects of Manganese Exposure- Human and Animals Models, Little Rock, AR, September 2006.

Antonini JM, Roberts JR, Sriram K, Benkovic SA, O'Callaghan JP, and Miller DB. Extrapulmonary tissue distribution of metals following repeated lung exposures to welding fumes with different elemental profiles. Society of Toxicology Annual Meeting, Seattle, WA, March 2008. Toxicol Sci: The Toxicologist, in press 2008.

Antonini JM, Stone S, Roberts JR, Schwegler-Berry D, Moseley A, Donlin M, Cumpston J, Afshari A, and Frazer DG. Pulmonary effects and tissue distribution of metals after inhalation of mild steel welding fume. American Thoracic Society International Conference, Toronto, Ontario, May 2008. Am J Respir Crit Care Med, in press 2008.

Conclusions

An animal model was developed that assessed the potential neurological responses associated with welding fumes that contained differing levels of manganese. Two methods of treatment were used to expose the laboratory animals to welding fumes: intratracheal instillation and inhalation. Intratracheal instillation is a method by which welding particles are collected onto filters during generation and directly instilled into the lungs of animals via the trachea after suspension in aqueous solution. It is simple, cheap, and large number of animals and treatment groups can be treated at one time. Also, the welding particles are directly administered to the distal alveolar regions of the lungs bypassing upper airway deposition (e.g., nasal/olfactory). Thus, translocation of metals after exposure from the respiratory system would be known to originate from the alveolar regions to the circulation and would not result from olfactory uptake. The advantages of inhalation exposure are the procedure is more physiological, deposition of the particles is more evenly distributed in the lungs, and the upper airways are involved, allowing assessment of possible olfactory transport of metal particles to brain areas. Unfortunately, inhalation exposure can be technically challenging and be quite expensive.

Our research group at NIOSH has developed an automated robotic welder to expose laboratory animals. The fume generated by our generator has been observed to be comparable to welding fume collected in the workplace. For this study, short-term inhalation exposures to GMA-MS welding fume, the most common in U.S. industries, were performed. Important findings from the short-term exposures indicate that manganese can translocate from the respiratory tract to other organ systems. Importantly, manganese was observed to deposit in the olfactory bulb within one hour after a 3-hour exposure to the fume. Due to the significant number of nanometer-sized particles (<0.1 um), it is possible that intact particles are being transported along olfactory nerve processes to the brain regions, bypassing the BBB. Interestingly, manganese levels quickly returned to control levels in the olfactory bulb by 24 hours after exposure. After this short-term exposure, no evidence of biochemical cellular damage or pathological changes were observed in the different brain regions. To make more definite conclusions about the potential neurotoxicity of GMA-MS fume, longer, more chronic

exposures are needed. Longer-term exposures to GMA-MS fume for 30 and 90 days have not been completed at this time.

Similar observations were made after exposing animals by the intratracheal instillation method with fumes containing differing levels of manganese. Manganese was found to translocate from the lungs via the circulation to other organs. There is evidence that manganese levels increased in the striatum after 7 weeks of treatment. Consistent with the observed accumulation of manganese in the brain, intratracheal instillation of MMA-HS (high Mn) or GMA-MS (low Mn) welding fumes for 7 or 11 weeks differentially elicited neuroinflammatory responses in the olfactory bulb, striatum, and midbrain, brain areas that are predominantly dopaminergic; the latter two being commonly affected in PD.

Current faculty receiving support from the grant:

No investigator on study received salary support.

References

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Mechanisms of Manganese-Induced Damage at the Cell and Mitochondrial Level

PRINCIPAL INVESTIGATOR:
(Enter the name and degree of Principal Investigator and any Associates)
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CONTRACTING ORGANIZATION:
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14. ABSTRACT

Oxidation rate results show that under State 3 conditions, Mn2+ inhibits the F1F0 ATP synthase of mitochondria, and that this inhibition is significant at a relatively low concentration of intra-mitochondrial Mn2+ (circa 10 nmoles/mg mitochondrial protein). Under similar conditions, Mn2+ does not inhibit mitochondrial electron transport complex I. Therefore, if Mn2+ has a direct role in inducing signs of idiopathic Parkinsonism, it does so through a different mechanism than MPP+ or rotenone.

15. SUBJECT TERMS

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					USAMRMC Thomas Gunter
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Introduction

In the Specific Aims for this grant, we proposed to use oxidation rate assays with isolated mitochondria 1) to determine whether Mn^{2+} interferes with Ca^{2+} activation of pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH), γ-ketoglutarate dehydrogenase (γ KGDH), and the F_1F_0 ATP synthase of mitochondria and 2) whether Mn^{2+} can inhibit mitochondrial electron transport complex I, we also proposed 3) to characterize the role of Mn^{2+} on ATP production, ROS production, and cell death in dopaminergic cell lines and astrocytes in the presence of factors which produce signs of Parkinsonism in animals. We now have important data regarding Specific Aims 1) and 2), and have developed the techniques which we need to carry out specific Aim 3).

Specifically, our oxidation rate results show that under state 3 conditions, Mn^{2+} does inhibit the F_1F_0 ATP synthase of mitochondria, but that under the same conditions, it does not inhibit mitochondrial complex I. The importance of the latter observation is that inhibitors of mitochondrial complex I, such as MPP^+ and rotenone have been implicated in inducing signs of Parkinsonism. While our results do not rule out a role for Mn^{2+} in inducing signs of Parkinsonism, they do suggest that if Mn^{2+} does this, it does not do it in the same way as MPP^+ or rotenone.

Body

In 1992, we showed that Mn^{2+} inhibited oxidative phosphorylation in mitochondria using simple oxidation rate experiments[1]. In 2003, Zwingmann et al also showed the same thing using several types of NMR[2]. These authors also suggested that the inhibition was at succinate dehydrogenase and perhaps pyruvate dehydrogenase. In the meanwhile, several groups had supported earlier work suggesting that within the cell, Mn is sequestered by mitochondria[1, 3-6], and we had used XANES spectroscopy (i.e. x-ray spectroscopy) to show that it was very unlikely that enough Mn^{3+} was produced by oxidation of Mn^{2+} by reactive oxygen species to cause toxic effects[7, 8, 9]. This meant that the most likely causes of Mn toxicity within the target tissue (globus pallidus and striatum) were from effects of Mn^{2+} at the mitochondrial level or from Mn^{3+} transported into the cell via the transferrin mechanism. Because of these results and the observation that Mn^{2+} always binds to Ca^{2+} binding sites, usually with an affinity significantly greater than that of Ca^{2+}, we hypothesized that a likely proximal cause of Mn toxicity was a drop in ATP production in the critical target tissue caused by Mn^{2+} interference with intramitochondrial Ca^{2+}'s most important role of activating the rate of ATP production by oxidative phosphorylation[10]. Intramitochondrial Ca^{2+} can activate the rate of ATP production by a factor of up to 2 or 3[11] and this can be very important for the function of active neurons in the basal ganglia. We knew from earlier work on the effects of intramitochondrial Ca^{2+}, that the intramitochondrial sites at which Ca^{2+} activates oxidative phosphorylation were PDH, ICDH, □KGDH and the F_1F_0 ATP synthase(for review see[12]. These then represented the likely sites at which Mn^{2+} might inhibit oxidative phosphorylation. Succinate dehydrogenase, the site identified by Zwingmann et al[2], is not a site at which Ca^{2+} activates ATP production and we were skeptical about it being a site at which Mn^{2+} inhibited the process.

The conditions under which the measurements are made are also important. Oxidative phosphorylation is carried out in cells between what are referred to as mitochondrial states 3

and 4 where the more energetically active the cell, the closer to state 3 conditions. The conditions that Zwingmann et al[2] used with cultured cells were far from state 3 conditions. Therefore, we wanted to use more incisive oxidation rate experiments under state 3 conditions in which the regions of the Krebs cycle and the electron transport chain, etc. that were activated were carefully controlled to try to determine where the inhibition of oxidative phosphorylation by Mn^{2+} occurred.

In addition to this, we had figured out a possible way in which Mn^{2+} could inhibit complex I of the mitochondrial electron transport chain (ETC). This was very interesting because inhibitors of complex I of the ETC such as rotenone and MPP^{+} are know to induce signs of Parkinsonism and Mn is suspected of being a risk factor for idiopathic Parkinsonism. We could use similar experiments to see if Mn^{2+} indeed did inhibit complex I of the ETC.

In the experiments focused on specific aims 1) and 2) we first used liver mitochondria and are now using heart and brain mitochondria. We began by showing that the oxidation rates were appropriate for state 3 conditions energized by succinate and by glutamate plus malate. HPLC is being used to verify that energization is through the expected parts of the Krebs cycle and ETC. Uncouplers are used to separate effects on the Krebs cycle and ETC from those on the F_1F_0 ATP synthase. Figure 1 shows the effects of Mn^{2+} on the oxidation rate when 6 mM succinate is used as substrate. There is a significant inhibition of the rate which increases with Mn^{2+} concentration. When the uncoupler DNP is added to the suspension to uncouple phosphorylation from oxidation there is no Mn^{2+} inhibition. This indicates that the inhibition is at the F_1F_0 ATP synthase. There is no other possibility. If there were inhibition at succinate dehydrogenase, then there would also be inhibition when the uncoupler, DNP is used. Similar results were obtained when glutamate plus malate were used as substrate. Figure 2 shows the relative rates of Mn^{2+} inhibition when mitochondria energized by succinate are compared with mitochondria energized by glutamate plus malate. There is essentially no difference in the rates or in the effects of Mn^{2+} on the rates. When mitochondria are energized using glutamate plus malate, the primary product is NADH which feeds reducing equivalents into ETC complex I, while when succinate is used as substrate only $FADH_2$ is produced which bypasses ETC complex I and feeds reducing equivalents into ETC complex II. The observation that the effects of Mn^{2+} inhibition are similar when reducing equivalents are fed into the ETC via complex I and via complex II tells us that the Mn^{2+} inhibition is not at ETC complex I. The conditions used do not test whether or not there is inhibition at PDH since this part of the Krebs cycle is not used when succinate or glutamate plus malate are used as substrates. Energization using pyruvate plus malate is rate limited by substrate transport in liver mitochondria and so with this system we cannot use these substrates to test Mn^{2+} inhibition at PDH; however, we should be able to carry out this experiment using heart or brain mitochondria and we are currently doing that.

Key Research Accomplishments

We have shown that:

1) Inhibitory effects of Mn^{2+} on oxidative phosphorylation can be seen with relatively small amounts of Mn^{2+} (5 to 10 nanomoles/mg mitochondrial protein).
2) The inhibition observed under state 3 conditions is at the F_1F_0 ATP synthase.
3) There is no inhibition observed under state 3 conditions at succinate dehydrogenase.

4) No inhibition of ETC complex I by Mn^{2+} can be observed under state 3 conditions. Therefore if Mn^{2+} contributes to induction of Parkinsonism, it does so differently from MPP^{+} and rotenone.

Reportable Outcomes

All of the results discussed above represent reportable outcomes and we plan to report them as soon as we finish additional experiments and further controls.

Conclusion

This work represents part of our test of our hypothesis that Mn^{2+} can interfere with Ca^{2+} activation of oxidative phosphorylation and that this may represent the proximal cause of Mn toxicity. We have found that Mn^{2+} does inhibit oxidative phosphorylation at the F_1F_0 ATP synthase. These results support the hypothesis.

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Appendices

See Figures 1 and 2.

Figures
Figure 1

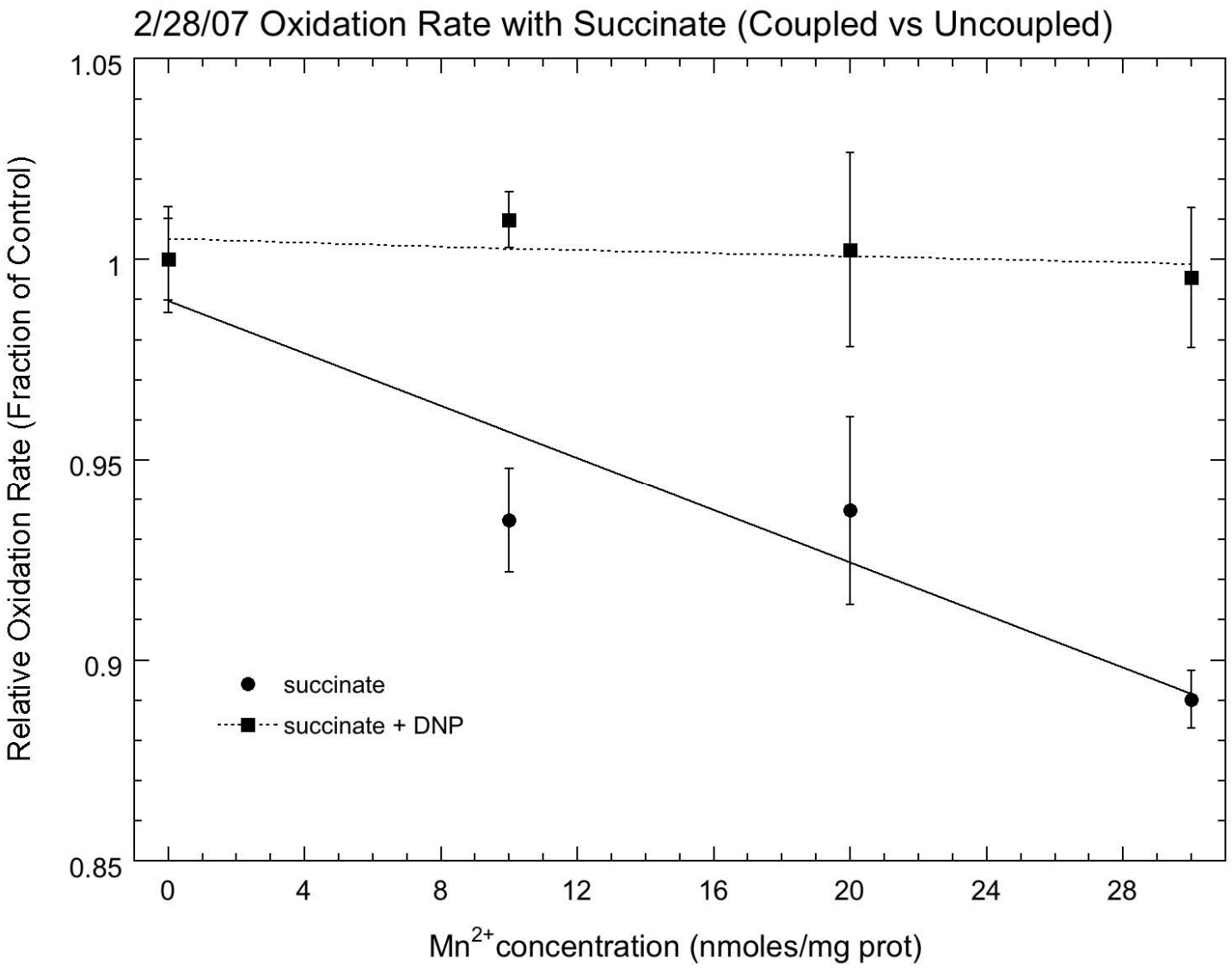
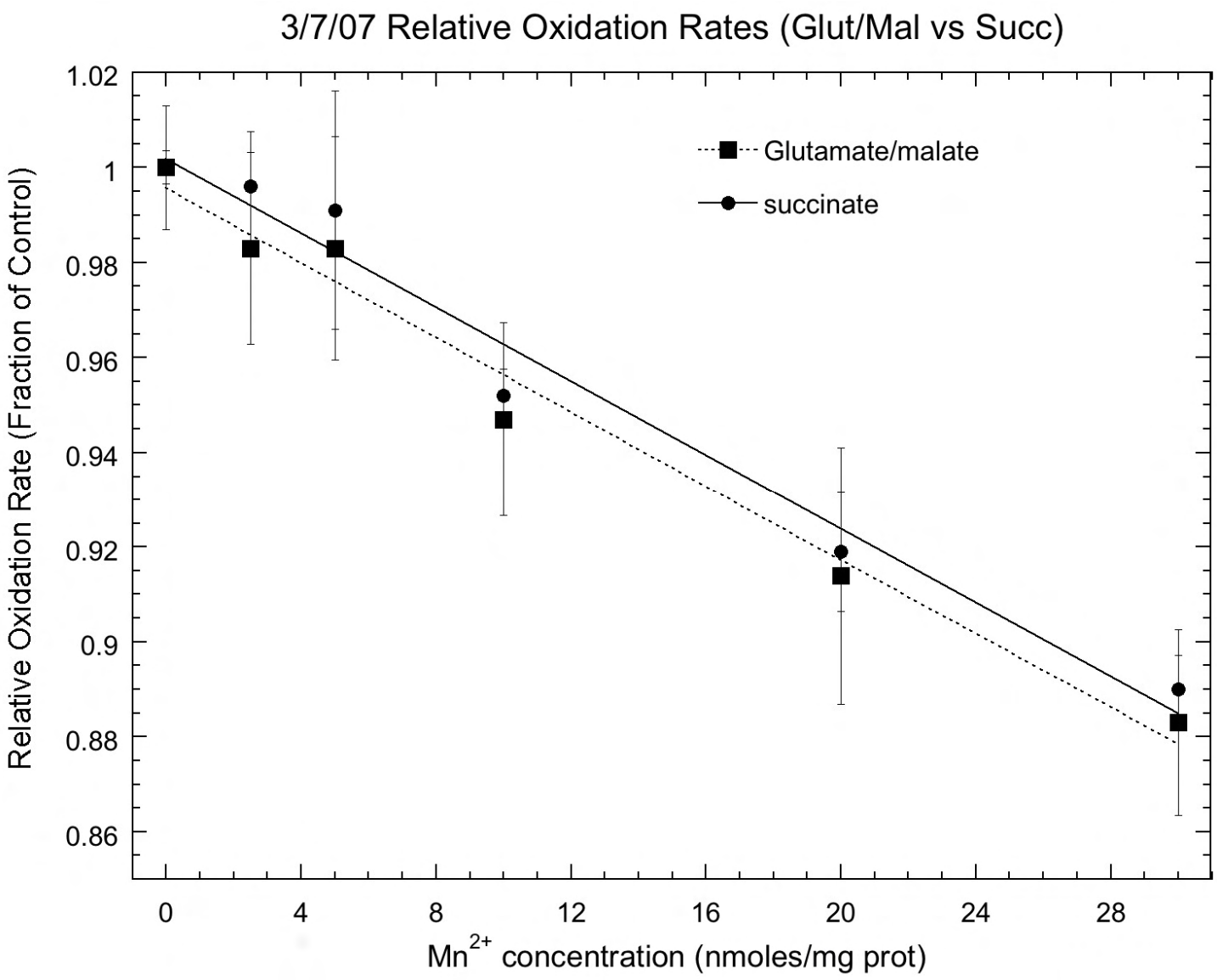


Figure 2


3/7/07 Relative Oxidation Rates (Glut/Mal vs Succ)
Glutamate/malate
succinate
Relative Oxidation Rate (Fraction of Control)
1.02
1
0.98
0.96
0.94
0.92
0.9
0.88
0.86
0
4
8
12
16
20
24
28
Mn2+ concentration (nmoles/mg prot)

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TITLE: MOLECULAR MECHANISMS UNDERLYING MN NEUROTOXICITY

PRINCIPAL INVESTIGATOR: Jane Wu, MD, PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
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14. ABSTRACT

Accumulating evidence supports that manganese (Mn) exposure leads to neurotoxicity and manganese-associated Parkinsonism. However, little is known about the mechanisms that contribute to manganese toxicity. Mn-neurotoxicity has not been well characterized at the cellular level. . We proposed to study **the molecular damages caused by Mn exposure using primary neuronal cultures and animal model. Using** primary mescencephalic neuronal cultures, we have examined effects of Mn treatment. Our study revealed that dopaminergic neurons are highly vulnerable to Mn exposure, with significant changes in cytoskeleton, in neurites and synapses. Mn treatment causes neurite fragmentation with aggregation of tau, beta-tubulin and alpha-synuclein proteins, and eventually apoptotic cell death of mescencephalic neurons. Even at a low concentration before eliciting cell death, Mn^{2+} treatment caused appreciable changes in tau distribution, suggesting that cytoskeletal infrastructure derangement is an early event in Mn^{2+} induced neurotoxicity.

15. SUBJECT TERMS
Manganese, neurotoxicology, iron deficiency, welding, manganese mining, nutrition

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Introduction

Although accumulating evidence supports that Mn exposure causes neurotoxicity. Molecular and cellular nature of Mn toxicity, in particular, the effects of Mn on synaptic structure and cytoskeletal integrity of dopaminergic neurons have not been characterized. We proposed to understand the molecular and cellular characteristics of neurotoxicity caused by Mn exposure.

Body

Examination of molecular and cellular changes of Mn neurotoxicity in primary mesencephalic neurons.

Mesencephalic cells were isolated from rats at embryonic day 16 or 17 (E16 or17). At different time points following establishment of culture, Mn was added at different concentrations. Cell morphological and molecular changes were examined using immunostaining followed by fluorescent microscopy. We examined dopaminergic neurons using TH as a marker. We examined cell morphology, neurite formation, cytoskeletal changes and expression of synaptic proteins using immunostaining with specific antibodies against α-synuclein, cytoskeletal proteins β-tubulin and tau-1, and synapsin1. We have also begun to examine neurotoxicity induced by Mn exposure in mice. Data analyses are in progress.

Key Research Accomplishments

Mesencephalic cells were isolated from rats at embryonic day 16 or 17 (E16 or17). At different time points following establishment of culture, Mn was added at different concentrations. Cell morphological and molecular changes were examined using immunostaining followed by fluorescent microscopy.

Comparison of control and Mn-treated groups show that dopaminergic neurons are highly vulnerable to Mn exposure, with significant changes in cytoskeleton, in neurites and synapses. Mn treatment causes neurite fragmentation with aggregation of tau, beta-tubulin and α-synuclein proteins, and eventually apoptotic cell death of mescencephalic neurons. Even at a low concentration before eliciting cell death, Mn^{2+} treatment caused appreciable changes in tau distribution, suggesting that cytoskeletal infrastructure derangement is an early event in Mn^{2+} induced neurotoxicity.

Reportable Outcomes

Manuscript:

Valentina Savchenko, BethAnn McLaughlin, Michael Aschner and Jane Wu. Manganese Induces Cytoskeletal Rearrangement and Apoptotic Death of Dopaminergic Neurons. (Manuscript in preparation)

Conclusions

Our data show that Mn exposure for 24-48hrs, even at relative low concentrations (100uM), is sufficient to cause neuronal damages. These include aggregation of neuronal proteins, redistribution of proteins such as α-synuclein protein, the loss of mesencephalic synapses , fragmentation of neuritis and finally neuronal cell death.

References

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14. ABSTRACT

Inhalation of high manganese (Mn) concentrations may result in serious irreversible neurological disease (manganism). Cases of manganism in welders are reported each year in Russia. Welding fumes contain Mn among many other components also Mn. Exposure to Mn in lower concentrations can result in motor system disturbances. The exposure level associated with an increased risk of acquiring these disturbances is currently not known. 150 welders are compared to 150 non-exposed referents in a cross-sectional study design. Neuro-behavioral tests are applied, parameters for iron status are measured and personal exposure assessed. Also 50 patients diagnosed with manganism are compared with 25 patients with idiopathic Parkinson's disease. 21 subjects will be examined with Positron Emission Tomography as well. All necessary preparations before sampling have been completed, and examinations of participants have started.

15. SUBJECT TERMS
Manganese, neurotoxicology, welders, manganism, Parkinson's disease

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Table of Contents

Introduction

Manganese (Mn) is an essential trace element in man. However, inhalation of high Mn concentrations has been associated with serious irreversible neurological disease (manganism). Cases of manganism are reported each year in Russia among welders. Welding fumes may contain high amounts of Mn. Welders are by number the most important group of workers occupationally exposed to Mn. Exposure to Mn in lower concentrations can result in subtle motor disturbances as well. The exposure level associated with an increased risk of acquiring such disturbances is currently not sufficiently known.

In this investigation 150 welders are compared to 150 non-exposed referents in a cross-sectional study design. Neurobehavioral tests are applied, parameters for iron status are determined and an extensive exposure assessment is also carried out. The main objective is to assess the value of these tools in an epidemiological study, in order to investigate their sensitivity for detecting subtle neurological functional changes. In addition 50 patients who have received the diagnosis of manganism will be assessed with the same clinical examinations and compared with 25 patients with newly diagnosed idiopathic Parkinson's disease (PD). A small subsample of 21 subjects (7 patients with manganism, 7 with PD and 7 referents will be examined with Positron Emission Tomography (PET-Scan) as well. Nearly 100 welders, 100 referents and 27 patients were studied in 2003, and thus a part of the study represents a follow up of these subjects. Results from that study have been published (Ellingsen et al., 2006; Ellingsen et al., 2007; Ellingsen et al., 2008).

Body

A contract was signed between the National Institute of Occupational Health (Norway) and Vanderbilt University (USA) on January 31, 2007, to carry out "A Study of the Nervous System in Welders". In the letter from Vanderbilt University Medical Center to the National Institute of Occupational Health in Norway dated March 8, 2007, the fully executed original of the contract was received, and this date represents the start of the project.

After the original contract was received, preparations for examining the participants were started. Sampling equipment for the collection of biological samples has been purchased. Air filters and air filter cassettes for the collection of personal samples of welding fumes in air have been purchased and transported to Russia from Norway. More than 300 filters have been weighed on a micro-weight to prepare them to be mounted into the filter cassettes as a preparation for the exposure assessment. The necessary equipment for conducting the neurobehavioral examinations was checked before shipment to Russia. However, software problems occurred with the CATSYS test system. This equipment is of fundamental importance for fulfilling the study aims as described in the study protocol. The CATSYS test system had to be transported back to the producer for adjustments in Denmark. These problems were finally settled, but caused some delay in the progress of the study. The delay in the progress of the study was about 3 months, which is not critical for conducting the study. All neurobehavioral equipment required for the study has been transported to Russia and is now ready for use.

Our neuropsychologist was in Russia for the final preparations before the examinations of the study participants can start. She has a supervision role for the testing, and videotapes were made for training and supervision/standardization purposes of the neurobehavioral testing. One critical part of the study is the collection of patients with newly diagnosed idiopathic Parkinson's

disease (PD). We have therefore concentrated on the examinations of PD patients at this stage of the study. The examination of participants to the study has started.

A continuing review of the study was conducted by the end of October 2008. A renewed continuing review date was established for October 1, 2008.

Key Research Accomplishments
Study subject identified
All testing will commence soon

Reportable Outcomes
None so far

Conclusion
All preparations prior to the examinations of participants have been completed.
Examinations of study subjects have recently started

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Ellingsen DG, Konstantinov R, Bast-Pettersen R, Merkurjeva L, Chashchin M, Thomassen Y, Chashchin V. A neurobehavioral study of current and former welders exposed to manganese. NeuroToxicology 2008;29(1):48-59.

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AWARD NUMBER: W81XWH-05-1-0239

TITLE: ROLE OF MANGANESE IN PRION DISEASE PATHOGENESIS

PRINCIPAL INVESTIGATOR: Anumantha Kanthasamy, Ph.D.

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Role of Manganese in Prion Disease Pathogenesis

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14. ABSTRACT

For the last two years, we systematically examined whether Mn interacts with prion protein to alter its cellular function. We first compared the effect of Mn on mouse neural cells expressing prion protein (PrP^{C}-cells) and lacking prion protein (PrP^{KO}-cells). Exposure to Mn produced a dose-dependent cytotoxic response in both PrP^{C}-cells and PrP^{KO}-cells. Interestingly, PrP^{C}-cells (EC_{50} 117.6 μM) were more resistant to Mn-induced cytotoxicity, as compared to PrP^{KO}-cells (EC_{50} 59.9 μM). Analysis of intracellular Mn levels showed less Mn accumulation in PrP^{C}-cells as compared to PrP^{KO}-cells. Furthermore, Mn-induced mitochondrial depolarization, ROS generation, and caspase-9 and -3 were significantly attenuated in PrP^{C}-cells as compared to PrP^{KO}-cells. Importantly, DNA fragmentation induced by Mn treatment was significantly suppressed in PrP^{C}-cells as compared to PrP^{KO}-cells. Furthermore, analysis showed an increase of PrP^{C} in both cytosolic and membrane-rich fractions after treatment with Mn. This increase in PrP^{C} levels relative to untreated cells was not due to increased mRNA transcription or protein degradation. Pulse-chase analysis showed that the PrP^{C} turnover rate was significantly altered with Mn treatment. Limited proteolysis using proteinase-K revealed that Mn treatment reduces the proteolytic rate of PrP^{C}. Together, these results demonstrate that Mn interacts with cellular prion protein and alters biological functions and protein stability. Current studies are underway to determine whether the stabilization of prion protein accelerates prion protein aggregation in scrapie infected cells treated with Mn.

15. SUBJECT TERMS
Manganese, neurotoxicology, prion diseases, oxidative stress, and protein aggregation

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Introduction

Prion diseases are fatal neurodegenerative diseases in both animals and humans. Although the etiology is unknown, aberrant processing of cellular prion proteins is well established in the pathogenesis of prion diseases. Normal cellular prion protein (PrP^{C}) is highly conserved in mammals and is expressed predominantly in the brain. Although the exact function of the normal prion protein in the CNS has not been fully elucidated, studies have suggested that the prion protein can function as a metal binding protein, an antioxidant, a cellular adhesion molecule and a signal transducer (Mouillet-Richard et al., 2000; Schmitt-Ulms et al., 2001; Chiarini et al., 2002; Nishimura et al., 2004). The four-six octapeptide repeat sequences toward the N-terminus of the protein have high affinity for divalent cations including copper, zinc, and manganese, reportedly in decreasing order of affinity as listed (Hornshaw et al., 1995; Brown et al., 1997; Viles et al., 1999; Garnett and Viles, 2003). Also, the brains of prion knockout mice had lower concentrations of these metals than the brains of normal mice (Brown, 2003). Further studies have shown that altered Mn content was observed in brain infected with the human prion disease known as Crueztfelt-Jacob Disease (CJD) (Wong et al., 2001). The pathological progression of many prion diseases including CJD is believed to be induced by conformational change in the structure of the normal prion protein (PrP^{c}) into an infectious prion particle (PrP^{sc}) (Prusiner, 1982). PrP^{sc} tends to aggregate into plaques which are highly resistant to digestion with proteinase K (Hay et al., 1987). Binding of Mn to the normal prion protein has been suggested to result in partial resistance to protease digestion and possibly conformational change in the infectious PrP^{sc} (Brown et al., 2000). Also, chronic Mn exposure may result in altered Mn binding to PrP^{c} and may increase the likelihood of conversion of PrP^{c} to the proteinase-resistant PrP^{sc}. Thus, accumulating evidence suggests that manganese content in the brain may play a crucial role in the pathogenesis of sporadic prion diseases.

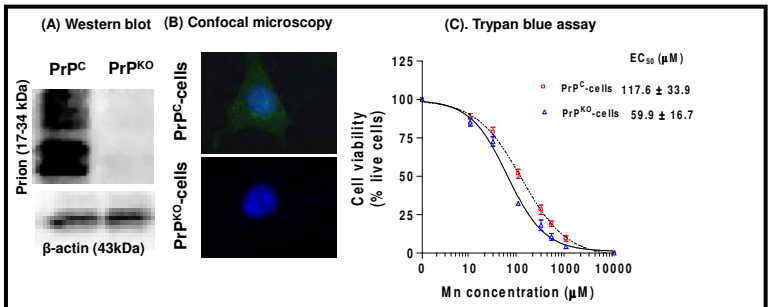


Fig. 1: Prion protein is expressed only in PrP^{c} cells and PrP^{C}-cells are more resistant to manganese toxicity. **(A)** Western blot was performed to verify prion protein expression in the PrP^{C}-cells, and the lack of prion protein expression in the PrP^{KO}-cells. β-actin was used to confirm equal loading of proteins. **(B)** Immunocytochemistry was also performed to verify prion protein expression in the cells. Prion protein was stained with anti-3F4 antibody conjugated with Alexa 488 (green stain). Nuclear staining was performed with Hoechst 33342. **(C)** Both PrP^{KO}- and PrP^{C}-cells were exposed to manganese (10-1000 μM), and cytotoxicity was measured using the Trypan blue exclusion assay.

Table 1: Increased uptake of manganese by PrP^{KO}-cells

Trace element analysis of internal manganese concentrations (ng/mg protein)

Treatment (6h)	PrP^{C}-cells	Fold Difference Compare to control	PrP^{KO}-cells	Fold Difference Compare to control
Untreated	2.4 0.6	1	0.25 ± 0.03 ***	1
30 μM Mn	112.9 ± 4.8	47.5	205.7 ± 6.4 ***	839.4
100 μM Mn	428.9 ±13.2	180.2	537.2 ± 16.7***	2192.5

Trace element analysis of internal copper concentrations (ng/mg protein)

Treatment (6h)	PrP^{C}-cells	Fold Difference Compare to control	PrP^{KO}-cells	Fold Difference Compare to control
Untreated	23.9 5.2	1	7.3 ± 3.0 ***	1
30 μM Mn	18.77 ± 8.0	0.8	4.3 ± 1.2 ***	0.6
100 μM Mn	27.0 ± 5.5	1.1	4.5 ± 1.1 ***	0.6

Values represent mean S.E.M. performed in n=4. (******P<0.001 for comparison between groups)***

STUDY 1 – Role of prion protein in Mn-induced neurotoxicity

The overall objective of this study was to determine the effect of manganese (Mn) on oxidative stress, mitochondrial function, cellular antioxidants, proteasomal function and protein aggregation in cell culture models of prion diseases. PrP^{c} has octapeptide repeat regions that bind to several divalent metals, suggesting that

the prion proteins may alter the toxic effect of environmental neurotoxic metals. Therefore, we systematically examined whether PrP^c modifies the neurotoxicity of manganese (Mn) by comparing the effect of Mn on mouse neural cells expressing PrP^c cells (PrP^c-cells) and prion-knockout cells (PrP^{ko}-cells).. Prion protein expression in PrP^c cells but not PrP^{ko} cells were confirmed in Western blot (Fig 1A) and confocal experiments (Fig 1B). Exposure to Mn (10 µM-1 mM) for 24 hr produced a dose-dependent cytotoxic response in both PrP^c-cells and PrP^{ko}-cells. Interestingly, PrP^c-cells (EC_{50}=60 µM) were more resistant to Mn-induced cytotoxicity, as compared to PrP^{ko}-cells (EC_{50}=112 µM), suggesting a protective role of PrP^c against Mn neurotoxicity (Fig 1C). Analysis of intracellular Mn levels by ICP-MS showed less Mn accumulation in PrP^c-cells as compared to PrP^{ko}-cells. However, PrP^c cells had a 9-fold higher basal level of Mn compared to PrP^{ko} cells, suggesting that the PrP^c cells retained Mn more effectively than PrP^{ko} cells (Table 1). This suggests that PrP^C may be involved in metal homeostasis, and the lack of the protein may result in lower than normal basal metal levels. Conversely, exposure to high concentrations of metals may result in reduced capability to prevent excessive metals from accumulating inside the cells. We also examined whether Mn exposure alters the levels of DMT-1 and transferrin in PrP^C-cells and PrP^{KO}-cells. Western blot analysis showed no significant differences in DMT-1 and transferrin between PrP^C-cells and PrP^{KO}-cells following 100µM Mn treatment, suggesting that the levels of these metal binding proteins do not account for the difference in Mn uptake in PrP^C-cells and PrP^{KO}-cells (Choi et al., 2007).

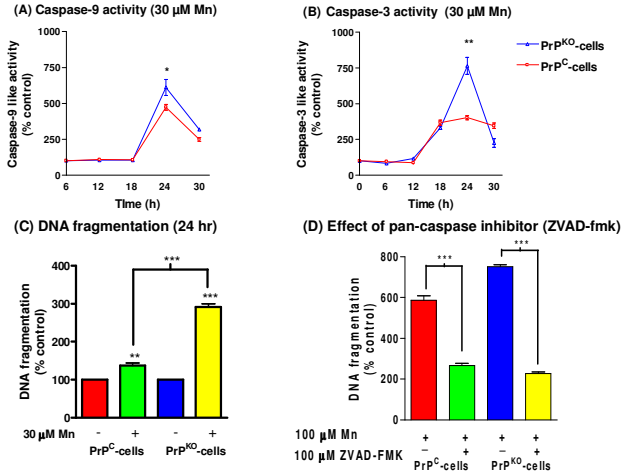


Fig. 4: Prion protein protects against Mn-induced caspase activation and apoptotic cell death. PrP^{KO} and PrP^C cells were treated with 30 µM or 100 µM Mn for 24 hr. (A) Caspase-9 and B) caspase-3 activities were measured in enzymatic assays using fluorescence substrates and microplate reader. (C) DNA fragmentation was measured using ELISA sandwich assay. (D) Cotreatment with pan-caspase-inhibitor ZVAD-fmk almost completely blocked Mn-induced increases in DNA fragmentation

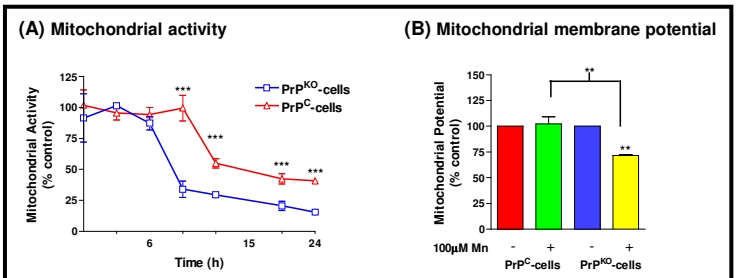


Fig. 2: Prion protein protects against manganese induced mitochondrial dysfunction. (A) Time course of mitochondrial activity. Cells were treated with 100µM Mn and mitochondrial activity was measured over a 24 h period using the MTT assay. (B) Mitochondrial membrane potential (ΔΨm) was measured using $DIOC_6$.

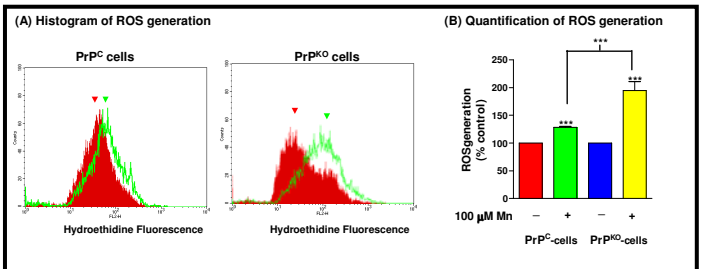


Fig. 3: **Prion protein protects against manganese-induced ROS generation (A)** Representative flow cytometric Histogram of dihydroethidium fluorescence in PrP^C-cells and PrP^{KO}-cells treated with 100 µM Mn. Dihydroethidium was added to the cells and incubated for 15 min at 37ºC in the dark. Then Mn was added, and fluorescence intensity was measured at 60 min by flow cytometry. The shift of the curve to the right indicates increase in ROS generation with Mn treatment as indicated by arrows. The x-axis shows the log scale of fluorescence intensity and the y-axis represents the cell count. For both histograms, the solid curve represents the untreated cells, while the outline represents the treated cells **(B)** Quantification of ROS generation with Mn treatment.

Furthermore, Mn-induced mitochondrial activity (Fig 2A), mitochondrial membrane potential (Fig 2B) and ROS generation (Fig 3), were significantly attenuated in PrP^c-cells as compared to PrP^{ko}-cells. To determine whether increases in ROS production are also accompanied by decreases in antioxidant GSH levels, we measured GSH levels initially in untreated PrP^{ko}- and PrP^c-cells. Baseline GSH

levels between these two cell lines did not differ significantly between these two cell lines, suggesting that PrP^c expression does not affect the basal intracellular GSH levels (data not shown). We then investigated whether Mn treatment alters GSH levels in PrP^{ko}- and PrP^c-cells. Exposure to 100 µM Mn for 24 hr decreased intracellular GSH levels in both PrP^c- and PrP^{ko}-cells (data not shown). Mn-induced ROS production and antioxidant depletion were followed by time- and dose-dependent activation of the apoptotic cell death cascade involving caspase-9 (Fig 4A) and caspase-3 (Fig 4B). Notably, Mn-induced DNA fragmentation was significantly suppressed in PrP^c -cells as compared to PrP^{ko}-cells (Fig 4C). Further, Mn-induced increase in DNA fragmentation was significantly attenuated by cotreatment with the pan-caspase inhibitor ZVAD-fmk suggesting that caspases-mediate Mn-induced apoptotic cell death (Fig 4D). Together, these results demonstrate that prion protein interferes with divalent metal Mn uptake and protects against Mn-induced oxidative stress and apoptotic cell death.

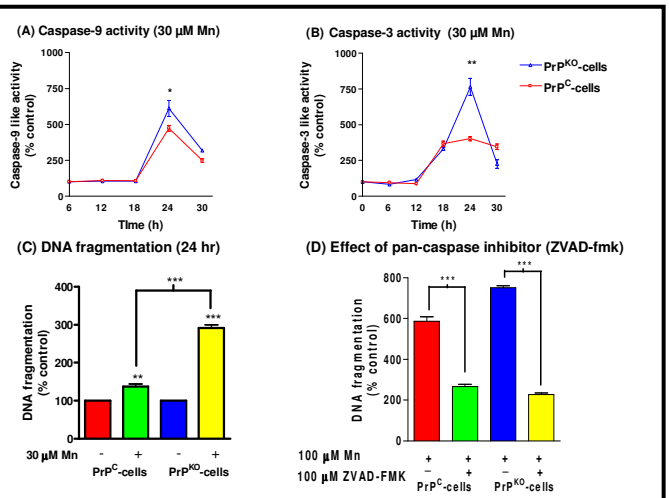


Fig. 4: Prion protein protects against Mn-induced caspase activation and apoptotic cell death. PrP^{KO} and PrP^C cells were treated with 30 µM or 100 µM Mn for 24 hr. (A) Caspase-9 and B) caspase-3 activities were measured in enzymatic assays using fluorescence substrates and microplate reader. (C) DNA fragmentation was measured using ELISA sandwich assay. (D) Cotreatment with pan-caspase-inhibitor ZVAD-fmk almost completely blocked Mn-induced increases in DNA fragmentation

Conclusions

We demonstrate for the first time that prion protein expression can rescue neural cells from Mn-induced apoptotic cell death, possibly by affecting intracellular Mn accumulation, as well as protect neural cells from oxidative damage. Finally, this study emphasizes the importance of characterizing the effect of metal interactions with cellular prion protein and elucidates a possible role of metals in the pathogenesis of prion diseases.

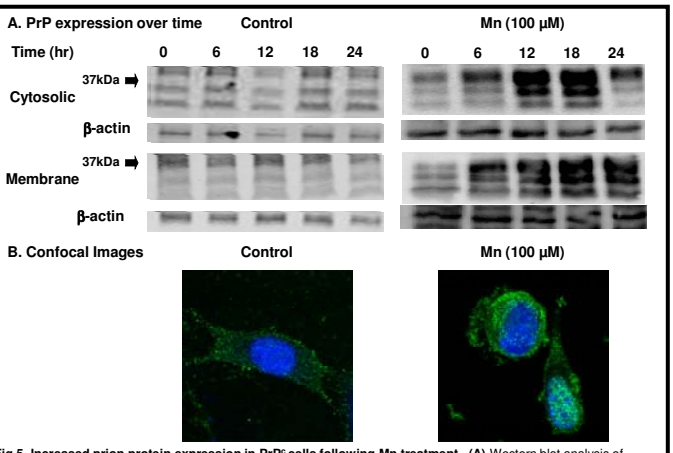


Fig 5. Increased prion protein expression in PrP^c cells following Mn treatment. **(A)** Western blot analysis of prion protein expression at various time points with manganese treatment. PrP^c cells were treated with Mn (100µM) for 0, 6, 12, 18, and 24 hr and harvested. Cytosolic fraction and membrane-rich fractions were obtained, and analyzed by Western blotting. **(B)** Immunocytochemical expression of prion protein with and without manganese treatment by confocal microscopy. PrP^C staining is in green, and nucleus staining is in blue.

STUDY 2 – Effect of Mn on PrP^c turnover, prion protein aggregation and proteinase K resistance In this study we examined whether Mn exposure alters cellular prion protein levels and prion protein aggregation and increases resistance to proteinase K (PK). We found that 100 µM manganese increases PrP expression in both cytosolic and membrane fractions in mouse PrP^c cells in a time-dependent manner (Fig. 5A). The untreated control cells did not exhibit any increase in PrP levels up to the 24 hr time point. Equal loading of protein was confirmed with β-actin. Fig. 5B shows the confocal images of prion protein expression in manganese-treated PrP^c cells. PrP staining is in green and nucleus staining is in blue. In addition to PrP^c cells, we also observed increased

accumulation of prion protein in N2A mouse neuroblastoma, as well as in Mn-treated mouse brain slices, indicating the toxicological importance of the study. Subsequently, we determined that upregulation of PrPc protein in Mn-treated cells was not due to increased transcriptional rate or the impaired ubiquitin proteasomal system. Since neither the transcriptional rate nor protein degradation was altered, we proceeded to investigate whether manganese altered the cellular metabolism of PrP. We measured the degradation rate of PrP^{c} in the presence or absence of manganese in a pulse-chase experiment (Fig 6A). The densitometric analysis of prion protein levels in the autoradiogram was evaluated at various chase time points. As shown in Fig. 6B, there was a significant difference in ^{35}S-labeled PrP between untreated and Mn-treated samples over a 24 hr chase period. The half-life of PrP in untreated samples was determined to be 13 hr, while the half-life was greatly increased to 21 hrs in manganese-treated samples. These results show that manganese significantly decreases the turnover rate of PrP, resulting in higher levels of PrP in manganese-treated cells relative to untreated controls.

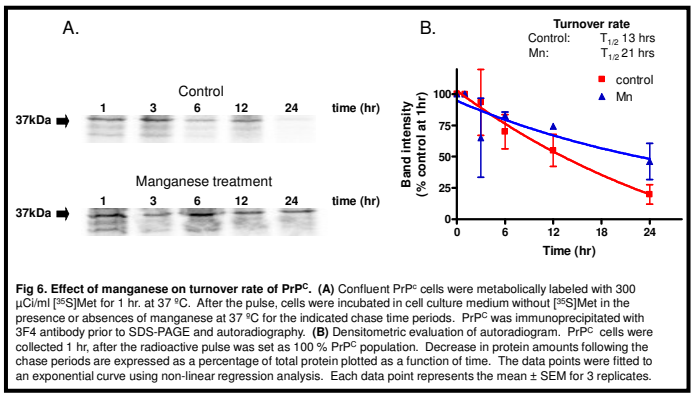
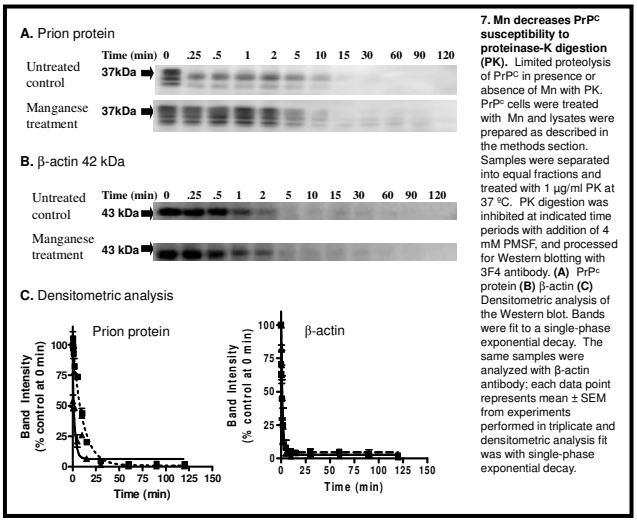


Fig 6. Effect of manganese on turnover rate of PrP^{c}. **(A)** Confluent PrP^{c} cells were metabolically labeled with 300 µCi/ml [^{35}S]Met for 1 hr. at 37 ºC. After the pulse, cells were incubated in cell culture medium without [^{35}S]Met in the presence or absences of manganese at 37 ºC for the indicated chase time periods. PrP^{C} was immunoprecipitated with 3F4 antibody prior to SDS-PAGE and autoradiography. **(B)** Densitometric evaluation of autoradiogram. PrP^{C} cells were collected 1 hr, after the radioactive pulse was set as 100 % PrP^{C} population. Decrease in protein amounts following the chase periods are expressed as a percentage of total protein plotted as a function of time. The data points were fitted to an exponential curve using non-linear regression analysis. Each data point represents the mean ± SEM for 3 replicates.

Next, we determined whether manganese treatment alters the proteolytic rate of prion proteins by performing a limited proteolysis assay with proteinase K (PK). PK-dependent proteolysis of prion protein is traditionally used for determination of the pathogenic form of PrP^{Sc} (Wong et al., 2001; Thackray et al., 2002). We compared the proteolytic susceptibility of PrP in manganese-treated cell lysates and untreated control lysates. The proteolytic rate of prion protein in manganese-treated cell lysates was slower than in untreated control lysates (Fig. 7A). To confirm the specificity of PK proteolysis, we immunoblotted the lysates with β-actin antibody and found no change in β-actin proteolytic susceptibility (Fig. 7B). These results suggest that the altered protease resistance of PrP in treated samples is a result of manganese binding to PrP, which stabilizes the protein. To further extend our research closer to the brain, we examined the effect of manganese on prion protein in mouse brain slice cultures. Interestingly, incubation of mouse brain slice cultures with manganese (300 µM) resulted in increased prion protein levels, and increased manganese content. Collectively, these data suggest that manganese increases the stability and accumulation of prion protein.



7. Mn decreases PrP^{c} susceptibility to proteinase-K digestion (PK). Limited proteolysis of PrP^{c} in presence or absence of Mn with PK. PrP^{c} cells were treated with Mn and lysates were prepared as described in the methods section. Samples were separated into equal fractions and treated with 1 µg/ml PK at 37 ºC. PK digestion was inhibited at indicated time periods with addition of 4 mM PMSF, and processed for Western blotting with 3F4 antibody. **(A)** PrP^{c} protein **(B)** β-actin **(C)** Densitometric analysis of the Western blot. Bands were fit to a single-phase exponential decay. The same samples were analyzed with β-actin antibody; each data point represents mean ± SEM from experiments performed in triplicate and densitometric analysis fit was with single-phase exponential decay.

In order to confirm the specificity of Mn on prion protein stability and PK resistance, we used cadmium, another divalent cation, as a negative control. Cd treatment did not result in an increase in the prion protein levels; however, Cd significantly inhibited proteasomal activity, resulting in the formation of high molecular weight ubiquitinated proteins, including higher levels

of ubiquitinated prion protein. Western blot and confocal analysis revealed that Cd treatment induced a dramatic increase in formation of soluble oligomers determined using A11 anti-oligomer antibody. Cd treatment also did not alter the PK-sensitivity of prion protein. The findings suggest that Mn specifically interact with prion protein to stabilize the protein.

Conclusions: The results from this study suggest that manganese increases the stability and accumulation of prion protein by altering the turnover rate of fully processed PrP^c through either indirect methods or in a manner requiring co-translational loading of manganese to PrP^c, thereby resulting in altered PrP^c biological half-life. Further, manganese treatment altered the PK sensitivity of PrP^c. Together, increased PrP^c stability and altered PK-resistance upon manganese exposure could accelerate the formation of aggregated PrP^{sc}. Confirmation of some of the results obtained with PrP^C cells in the N2A mouse neuroblastoma cell line and mouse brain slices indicates the toxicological importance of the study.

STUDY 3 – Effect of manganese in scrapie-infected neuronal cell models In this study we examined the effects of Mn in scrapie-infected cell models. We investigated Mn neurotoxicity in two different cell models of prion diseases, N2A (mouse neuroblastoma) and SN56 (mouse cholinergic cells). Uninfected and scrapie-infected N2A cells were purchased from InPro Tech (San Francisco CA), and uninfected SN56 and the RML strain infected $SN56^{sc}$ were obtained from Dr. Byron Caughey's laboratory at Rocky Mountain NIH Labs, Hamilton, MT (Baron et al., 2006) . First, we tested the prion infectivity in scrapie-infected N2A and SN56 cells using the PK digestion procedure. For PK digestions, the samples were incubated with PK (20µg/ml) at 37ºC for 30 min, followed by addition of PMSF (final concentration 2 mM) to stop digestion. After sample preparation, they were loaded onto 15% SDS-PAGE gels and resolved. One blot was probed with 8G8 monoclonal prion antibody (detects 95-110 residue in prion protein), and the other was probed with SAF32 monoclonal prion antibody (detects octapeptide repeat regions). In ScN2A and ScSN56 cells, 8G8 immunoblotted membrane showed distinct PK-resistant prion protein in both the scrapie fibril enrichment protocol and the TCA protocol. This was expected since PK digestion cleaves the N-terminus of the prion protein, leaving the protease resistant core (usually residues 90 and above). No PK-resistant bands were detected in non-infected SN56 and N2A cells. In the next set of studies, we examined whether scrapie infected N2A and SN56 cells retain their infectivity over different passages. The results revealed that N2A-infected cells lost their infectivity over time, whereas scrapie-infected SN56 cells retained the infectivity. Based on these results, we selected scrapie-infected SN56 cells over N2A cells for studies.

Microscopic visualization revealed that scrapie-infected $SN56^{sc}$ cells tends to aggregate into cellular clumps, whereas uninfected SN56 cells exhibited more of a mono-layer cellular morphology. We examined the relative susceptibility of $SN56^{sc}$cells and uninfected SN56 to manganese treatment. Cells were placed in 96 well plates at a density of approximately 2×10^4 cells per well. After 24hr, cells were treated with various doses of Mn, and relative mitochondrial activity was measured utilizing the MTT assay. The results showed that $SN56^{sc}$cells were sensitive to Mn-induced cytotoxic cell death as compared to uninfected SN56 cells. Further studies on the effect of Mn on mitochondrial function, protein aggregation, and proteasomal function in scrapie-infected $SN56^{sc}$ cells are underway. These studies will help us to understand the role of Mn in the pathogenesis and progression of prion diseases.

Conclusions: Our results indicate that the scrapie-infected $SN56^{sc}$ cell line is an optimal model for chronic Mn exposure studies. Preliminary results indicate that scrapie-infected SN56 cells are more sensitive to Mn-induced cell death as compared to uninfected cells. Further

studies on prion protein processing and stability in scrapie-infected SN56^{sc} cells lines will provide insights into cellular mechanisms of prion protein aggregation as well as the possible pathogenesis of prion disease.

Data analysis and statistics Data were analyzed with Prism 4.0 software (GraphPad Software, San Diego, CA). Bonferroni's post-hoc multiple comparison testing was used to delineate significance between different treatment groups. For densitometric analysis of limited proteolysis, band intensity was normalized to control bands at 0 min, and single-phase exponential decay was fit to the data. $P<0.05$ was considered significant, and differences are indicated with asterisks.

Key Research Accomplishments

- Mouse neural cells expressing prion (PrP^{c}) and lacking prion protein (knockout PrP^{ko}) were established as cell culture models of prion disease.
- Both these cell types were assessed for Mn-toxicity.
- Mn-induced mitochondrial depolarization, ROS generation, and caspase-9 and -3 activation were significantly attenuated in PrP^{c}-cells as compared to PrP^{ko}-cells.
- Basal Mn levels in PrP^{c}-cells were significantly different from levels in PrP^{ko}-cells.
- Mn-uptake was significantly reduced in PrP^{c}-cells as compared to PrP^{ko}-cells.
- Mn exposure did not alter the DMT1 and transferrin levels.
- Prion protein was neuroprotective against Mn-induced cytotoxicity and apoptotic cell death in cell culture studies.
- Mn upregulated prion protein expression in PrP^{c} and neuroblastoma N2A cells and in mouse brain slices.
- Mn upregulation of prion protein expression was independent of transcriptional activation and proteasomal degradation.
- Mn altered PrP^{c} protein turnover rate in pulse chase experiments, resulting in increased levels of PrP^{c}.
- Mn reduced the proteinase-K dependent proteolytic rate of PrP^{c}, suggesting increased PK-resistance.
- The stability effect of Mn in prion cell culture models was specific, because another divalent metal, Cd, did not cause similar effects.
- Scrapie-infected SN56 cells (SN56^{Sc}) served as a good infectious cell culture model for assessing the role of Mn neurotoxicity in prion diseases.

Reportable Outcomes

Manuscripts/Abstracts

- Choi, C.J., Kanthasamy, A., Anantharam, V., Kanthasamy, A.G. (2006), Interaction of metals with prion protein: possible role of divalent cations in the pathogenesis of prion diseases*. Neurotoxicol.,* 27: 777-87.

- Choi, C.J., Anantharam, V., Saetveit, N.J., Houk, R.S., Kanthasamy, A., Kanthasamy AG. (2007), Normal cellular prion protein protects against manganese-induced oxidative stress and apoptotic cell death. *Toxicol. Sci*., 98: 495-509.

- Choi C. J., Anantharam, V., Nicholson, EM., Richt, J.A, Kanthasamy, A. and Kanthasamy, A.G (2008). Manganese Upregulates Cellular Prion Protein and Inhibits the Rate of Proteinase-K-Dependent Limited Proteolysis in Neuronal cells. *Submitted for publication.*

- Choi C. J., Anantharam, V.,Kanthasamy, A. and Kanthasamy, A.G. (2008) Cadbium, not Manganese, impairs Neuronal Proteasomal Systems Leading to Formation of Protein Aggregation and Ubiquitinated Prion Proteins. *Manuscript in preparation.*

- Choi, C.J., Anantharam, V., Kanthasamy, A., & Kanthasamy, (2005). Neurotoxic Effect of Manganese and Neuroprotective Effect of Copper in a Cell Culture Model of Prion Diseases. A.G. Abstract No. 633. 44th Annual Meeting of Society of Toxicology, March 6-10, 2005, New Orleans, LA.

- Choi, C.J., Anantharam, V., Kanthasamy, A., & Kanthasamy, A.G. (2005). Effect of Prion Protein on Manganese-induced oxidative insult and mitochondrial Dysfunction. Abstract No. P101. Environment and Neurodevelopmental Disorders, Neurotoxicology Conference Series, September 11-14, 2005, Research Triangle Park, NC.

- Choi, C.J., Anantharam, V., Kanthasamy, A., & Kanthasamy, A.G. (2006). Effect of Divalent Metals Manganese and Cadmium on Ubiquitin-Proteasome Function and Protein Aggregation in Cell Culture Models of Prion Disease: Possible Role of Metals in Prion Disease Pathogenesis. 45th Annual Meeting of Society of Toxicology, March 5-9, 2006, San Diego, CA.

- Choi C. J., Anantharam, V., Nicholson, E.M., Richt, J.A, Kanthasamy, A. and Kanthasamy, A.G (2006). Manganese upregulates cellular Prion proteins and inhibits the rate of Proteinase-K dependent limited Proteolysis in Neuronal cells. 36th Annual Meeting of the Society for Neuroscience, Oct. 14-18, 2006, Atlanta, GA.

- Kanthasamy, A.G., Choi, C.J., Anantharam, V and Kanthasamy, A (2007) Manganese Exposure Enhances Prion Protein Turnover and Proteinase-K Resistance: Possible Role in Pathogenesis of Prion Diseases. SETAC Europe 17th Annual Meeting, 20 - 24 May 2007, Porto, Portugal.

Current faculty receiving support from the grant:

- Anumantha Kanthasamy, Ph.D.
- Vellareddy Anantharam, Ph.D.
- Arthi Kanthasamy, Ph.D.

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TITLE: Manganese Research Health Project (CFDA No. 12.420): Review of the literature on the genotoxicity and carcinogenicity of inorganic manganese compounds

PRINCIPAL INVESTIGATOR: Prof L.S Levy OBE

CONTRACTING ORGANIZATION: Institute of Environment and Health, First Floor, Building 63, Cranfield University, Cranfield, Bedfordshire MK43 0AL, UK

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14. ABSTRACT
This review was undertaken to establish understanding of the carcinogenic and mutagenic potential of manganese. Based on a structured comprehensive search, all relevant papers were identified and critically reviewed. Thus the assessment was designed to provide a systematic assessment of the robustness of individual experimental studies (considered against current OECD and other test method guidance and classification schemes) and to also consider the significance of the individual findings and overall knowledge base.
It was concluded that although there is some evidence that manganese may be a weak mutagen *in vitro* and possibly a genotoxicant *in vivo*, the underlying mechanisms are uncertain. The available data on carcinogenicity suggests that there is insufficient evidence that inorganic manganese causes cancer in animals or in humans.

In order to address the identified areas of uncertainty, it is suggested that additional research might be considered are:

- Mechanistic study(s) of the underlying processes involved in the *in vitro* genotoxic activity of inorganic manganese
- A short-term study to clarify the rodent clastogenic potential of manganese compounds.
- Mechanistic study(s) into basis for the interspecies differences in thyroid toxicity between mice and rats.

The draft report is currently undergoing indpeendent review by internationally recognised toxicological experts before formal submission of the report to the sponsor. The final report will provide a state of the art assessment of the current knowledge base with regard to the mutagenic and genotoxic potential of manganese, and will identify a potential research programme to address any remaining areas of uncertainty that are identified. Thus, this work is expected to influence future commissioning of research in these areas of toxicity.

15. SUBJECT TERMS
Manganese; Inorganic; Carcinogenicity, Mutagenicity, Genotoxicity

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					Leonard Levy, PhD

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Introduction

While the main route of exposure of the general population to inorganic forms of manganese is via their diet, in occupationally-exposed humans, inhalation of manganese is likely to be an important additional route.

With the exception of the well-documented neurotoxic effects of chronic manganese uptake on the central nervous system, manganese and its compounds are generally considered relatively non-toxic, and to have low mutagenic potential compared with some heavy metals. In particular, the carcinogenicity and mutagenicity of manganese compounds was reviewed by Gerber *et al*. in 2002 and again by the Institute of Environment and Health (IEH) in 2004. This latter review suggested that the knowledge base on the genotoxicity of inorganic manganese was incomplete and conflicting, and that there was only limited information on its carcinogenic potential. As a consequence, this up-date review of all the available published studies on the carcinogenic and genotoxic potential of inorganic manganese was undertaken by IEH (organic manganese compounds were, however not included). The review focused on the systematic assessment of the robustness of individual experimental studies (considered against current OECD and other test method guidance and classification schemes), as well as consideration of the significance of the individual findings and overall knowledge base.

Body

OVERALL OBJECTIVES

The objectives of this research project is to:

- Assess the strength of the existing evidence on the mutagenicity and carcinogenicity of manganese.
- Identify any gaps in knowledge or areas of uncertainty that prevent full characterization of the hazard potential of manganese with regard to these particular endpoints.
- Make recommendations for any further testing and/or guidance on the interpretation and potential consequences of possible outcomes of any proposed test strategy.

METHOD

Hard copies of all relevant papers identified in the previous review by IEH (IEH, 2004) were obtained. These were supplemented by further literature searches performed on the host Dialog DataStar, in Medline (for 1966+), Embase (1974+), Pascal (1990+), Biosis (+1969) and Toxfile (1966+) in May 2007 in order to identify any documents published during or after 2004. The search strategy was designed to specifically retrieve those papers that relate to a wide range of potentially relevant endpoints (including toxicity, carcinogenicity, mutagenicity or health effects of manganese). The search terms used to identify papers relating to inorganic manganese substances are detailed in Table 1, the CAS Numbers used in the search are detailed in Table 2, and the terms used relating to toxicity are listed in Table 3.

Truncation of search terms was used where appropriate, and papers identified using the search terms included in Table 1 and 2 were combined using the Boolean operator ‘OR’. The results from these sets were then combined with those obtained using the toxicity search terms (Table 3) using the Boolean operator ‘AND’. The title and, where available, abstract details were reviewed by one or more experienced toxicologists and those papers judged of potential relevance were obtained in hard copy.

Hard copies of relevant papers identified by the extensive search of the published and grey literature were obtained and systematically reviewed by experienced toxicologists with regards to the quality of methodology employed and the clarity of reporting; papers previously considered in IEH (2004) were subject to detailed reassessment. Throughout the review phase, particular attention was paid to assessing the similarity, or otherwise, to current OECD or other recognised test guidelines, the clarity of reporting, and the significance of the outcomes.

Table 2.1 Terms used for substances (searched for in title abstract and descriptors)

Braunite
Cianciulliite
Ferromanganese or ferro manganese – FeMn
Ferrosiliconmanganese or ferro silicon manganese
Manganese ore$1
Manganese oxide$1
Manganese sulphate or manganese sulphate
Manganese with steel – (title, abstract)
Manganous salt$1
Manganous Manganic Oxide or Hausmannite – Mn_3O_4
Polianite
Pyrochroite
Pyrolusite (manganese oxide)
Ramsdellite (manganese oxide)
Siliconmanganese or silicon manganese
Sodium manganate – Na_2MnO_4
Manganese
Manganese carbonate – $MnCO_3$
Manganese chloride or Manganese (II) chloride – $MnCl_2$
Manganese (III) fluoride – MnF_3
Manganese oxide or Manganese tetroxide – Mn_3O_4
Manganese (II) oxide – MnO
Manganese (III) oxide – Mn_2O_3
Manganese dioxide or Manganese (IV) oxide – MnO_2
Manganese nitrate or Manganese (II) nitrate – $Mn(NO_3)_2$
Manganese sulphate or Manganese (II) sulphate – $MnSO_4$
Manganese sulphide or Manganese (II) sulphide – MnS
Manganese oxide – MnO
Barium manganate – $BaMnO_4$
Potassium manganate – K_2MnO_4
Potassium permanganate or Potassium (VII) manganate –$KMnO_4$

$ (wildcard) retrieves up to the specified number of characters

Table 2.2 CAS Numbers included in search strategy

Substance	CAS No.
Manganese	7439-96-5
Manganese carbonate – $MnCO_3$	598-62-9
Manganese chloride tetrahydrate	13446-34-9
Manganese chloride or Manganese (II) chloride – $MnCl_2$	7773-01-5
Manganese (III) fluoride – MnF_3	7783-53-1
Manganese oxide/Manganese tetroxide – Mn_3O_4	1317-35-7
Manganese (II) oxide – MnO	1344-43-0
Manganese (III) oxide – Mn_2O_3	1317-34-6
Manganese dioxide or Manganese (IV) oxide – MnO_2	1313-13-9
Manganese nitrate or Manganese (II) nitrate – $Mn(NO_3)_2$	10377-66-9
Manganese (II) nitrate hydrate	15710-66-4
Manganese sulphate or Manganese (II) sulphate – $MnSO_4$	7785-87-7
Manganese sulphide or Manganese (II) sulphide – MnS	18820-29-6
Manganese oxide – MnO	1344-43-0
Barium manganate - $BaMnO_4$	7787-35-1
Potassium manganate – K_2MnO_4	10294-64-1
Potassium permanganate or Potassium VII manganate -$KMnO_4$	7722-64-7

Table 2.3 Search terms used for Toxicity in databases

Medline, Toxline	Embase	Biosis, Pascal
Carcinogen$5.ti,de,ab.	Carcinogen$5.ti,de,ab.	Carcinogen$5.ti,de,ab.
Tumor-markers-biological#	Carcinogen-testing#	Mutagen$5.ti,de,ab.
Carcinogenicity-tests#	Carcinogenic-activity#	Genotoxic$5.ti,de,ab.
Carcinogens-environmental#	Carcinogen-dna-interaction#	Cytotox$5.ti,de,ab.
Mutagen$5.ti,de,ab.	Mutagen$5.ti,de,ab.	Epidemiology.ti,de,ab.
Mutagenecity-tests#	Mutagenic-agent#	
Genotoxic$5.ti,de,ab.	Chemical-mutagen#	
dna-damage#	Promutagen#	
Cytotox$5.ti,de,ab.	Mutagen-testing#	

Epidemiologic-factors#	Chemical-mutagenesis#	
Epidemiologic-methods#	Environmental-mutagen#	
Epidemiology#	Mutagenic-activity#	
Effect-modifiers-epidemiolgy#	Genotoxic$5.ti,de,ab.	
Epidemiolog$2.ti,de,ab.	Cytotox$5.ti,de,ab.	
	Cytotoxic-agent#	
	Cell-mediated-cytotoxicity#	
	Cytotoxicity-test#	
	Epidemiology#	
	Cancer-epidemiology#	

Ti Title; de Descriptor; ab Abstract; $ (wildcard) retrieves up to the specified number of characters; # Descriptor only

FINDINGS

The current literature indicates that manganese may be a weakly mutagenic *in vitro* and possibly genotoxic *in vivo*. The mechanisms underlying these effects are uncertain, although possible roles for reactive oxygen species or interactions of manganese ion with DNA polymerases have been suggested. Available studies on the genotoxic effects of manganese in humans suffer from confounding as a result of co-exposure to other agents, thus it is not possible to determine whether manganese exposure could result in genotoxic effects in humans.

A study in rodents found equivocal evidence of manganese carcinogenicity in male and female mice, based on a slight increased incidence of thyroid tumours, but no evidence in rats. The significance of rodent thyroid cancers to human risk assessment has been widely questioned and since the thyroid effect was observed only in mice, the significance to humans is questionable. Furthermore, the available epidemiological studies do not provide any clear evidence that either occupational or environmental exposure to inorganic manganese is associated with a higher cancer risk. Given this, there is insufficient evidence that inorganic manganese causes cancer in animals or in humans.

The dataset on inorganic manganese, though not extensive, provides data of sufficient quality to permit a provisional assessment of the overall mutagenic, genotoxic and carcinogenic potential of the inorganic manganese compounds considered here. *In vitro* data on the mutagenicity and genotoxicity profile of manganese suggests that it is weakly mutagenic and genotoxic. However, in general effects have been observed at higher exposure levels than those required with other genotoxic metals and in general *in vitro* effects appear to associate only with cytotoxic levels. The mechanisms underlying the effects are poorly understood, although various potential mechanisms (such as generation of ROS or inhibition of DNA polymerases) have been suggested. Understanding the basis for the genotoxicity is a possible area warranting further investigation. Although there is some evidence to suggest that inorganic manganese may be clastogenic, the data do not support *in vivo* mutagenic potential. A well designed short-term

study to assess the clastogenicity of a number of representative manganese compounds in rodents might be considered to better define the genotoxic potential.

With regard to the carcinogenic potential of manganese, the relevance of the observed thyroid neoplasia in mice, but not rats treated in the NTP (1993) study, is uncertain. Thus it might be useful to undertake a study designed to elucidate the underlying mechanism(s) for the species difference and identify any thresholds that may exist and confirm if there is any potential relevance to humans.
Regarding the possibility of conducting further epidemiological studies, while there is currently insufficient evidence to confirm whether manganese is, or is not, genotoxic and/or carcinogenic to humans, carrying out robust occupational epidemiological studies free from confounding due to co-exposure to established genotoxic and/or carcinogenic metals or other substances would be difficult, at least until specific and robust biomarkers of exposure enabling the establishment of dose-response relationships become available. Similarly, it is considered unlikely that short-term human volunteer studies would inform on the long-term occupational situation. Thus, in summary, the areas where additional research might be considered are:

- Mechanistic study(s) of the underlying processes involved in the *in vitro* genotoxic activity of inorganic manganese
- A short-term study to clarify the rodent clastogenic potential of manganese compounds
- Mechanistic study(s) into basis for the interspecies differences in thyroid toxicity between mice and rats.

While the dataset for manganese is incomplete and, in some instances, not particularly robust, the need to conduct extensive further testing is considered questionable within the context of chemical regulation. There is currently insufficient data to establish manganese as a human carcinogen and, as discussed previously, it is unlikely that confirmation would be easily achieved from human studies.

While it is expected that additional mechanistic studies would enable further interpretation of the apparent range of genotoxic activities of inorganic manganese compounds, it is unlikely that this information would impact significantly on the overall regulatory assessment, hence such studies may not be considered as a high priority. However, investigation of the processes underlying the thyroid changes in mice would likely confirm whether the tumours occurred as a consequent of direct genotoxic effects or through perturbations of thyroid hormone homeostasis. Such studies would be useful in assessing the relevance of these thyroid effects to humans. For example, if manganese were shown to act through a genotoxic mechanism then it would be considered as a potential human carcinogen (COM, 2000). However, if the mechanism were shown to be of doubtful relevance to humans and a threshold of effect demonstrated, then it could be argued that the relevance to humans was low, although manganese may still be considered as a potential human carcinogen.

Therefore, the studies of greatest priority when considering additional research on the genotoxicity and carcinogenicity of inorganic manganese can be limited to:

- Mechanistic study(s) into basis for the interspecies differences in thyroid toxicity between mice and rats.

IMPACT

The draft report is currently undergoing indpeendent review by internationally recognised toxicological experts before formal submission of the report to the sponsor. The study will thus provided a state of the art assessment of the current knowledge base with regard to the mutagenic and genotoxic potential of manganese, and will identify a potential research programme to address any remaining areas of uncertainty that are identified. Thus, this work is expected to influence future commissioning of research in these areas of toxicity.

Key Research Accomplishments

This study has provided a state of the art assessment of the current knowledge base with regard to the mutagenic and genotoxic potential of manganese, and has identified a potential research programme to address the remaining areas of uncertainty.

Reportable Outcomes

None to date, though the intention is that the final report to the sponsor is either published on the web, or as a paper in modified form.

Conclusions

Although there is some evidence that manganese may be a weak mutagen *in vitro* and possibly a genotoxicant *in vivo*, the underlying mechanisms are uncertain. The available data on carcinogenicity suggests that there is insufficient evidence that inorganic manganese causes cancer in animals or in humans

References

Gerber, G.B., Léonard, A. & Hantson, P. (2002) Carcinogenicity, mutagenicity and teratogenicity of manganese compounds*. Critical Reviews in Oncology/Hematology,* 42(1), 25-34.

IEH (2004) *Occupational exposure limits: Criteria document for manganese and inorganic manganese compounds (Web Report W17*), Report by Institute for Environment and Health/ Institute of Occupational Medicine, Published: Leicester, UK, MRC Institute for Environment and Health (Available: http://www.silsoe.cranfield.ac.uk/ieh/publications/publications.html).

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AWARD NUMBER:

TITLE: Role of Toxins and Genetics in Manganese-Induced DA Neuron Degeneration

PRINCIPAL INVESTIGATOR: Richard Nass, Ph.D.

CONTRACTING ORGANIZATION: Indiana University School of Medicine, Indianapolis, IN

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14. ABSTRACT
A significant hindrance in dissecting the molecular components of Mn^{2+}-induced neurotoxicity is the high complexity of the vertebrate brain and lack of facile *in vivo* genetic models to determine and explore the mechanisms involved in the cell death. We have developed a novel pharmacogenetic model using the genetically tractable nematode *C. elegans* to dissect and characterize the molecular components involved in DA neuron degeneration (see Nass et al, PNAS, 2002; Nass and Blakely, Ann. Rev. Toxicol. Pharmacol., 2003). At the molecular level, the *C. elegans* nervous system is highly conserved both genetically and functionally with mammals, and all the genes responsible for DA biosynthesis, packaging, and reuptake are present and functional in the worm. We have shown that the nematode *C. elegans* DA neurons can be selectively damaged by exposure of whole animals to the parkinsonian-inducing neurotoxin 6-hydroxydopamine (6-OHDA) (see Nass et al, PNAS, 2002). We have also recently shown that a brief exposure to Mn^{2+} causes DA neuron cell death in the worm, and that prior exposure to Mn^{2+} amplifies the 6-OHDA-induced DA neurodegeneration. In our model system, the expression of the green fluorescent protein (GFP) in DA neurons will allow us a facile and powerful test to examine the role that DA, its metabolites, endogenous proteins, and neurotoxins play in Mn^{2+} -induced degeneration of DA neurons *in vivo*. These studies will also include a genome-wide screen to identify mediators and suppressors of Mn^{2+}-induced toxicity that will facilitate the identification of novel genes and molecular pathways involved in this highly relevant health and environmental concern.

15. SUBJECT TERMS
Role of Toxins and Genetics in Manganese-Induced DA Neuron Degeneration

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Introduction

The goals of the award are to identify and characterize the molecular pathways and mechanisms involved in manganese (Mn)-induced dopamine (DA) neuron cell death. Due to the many significant similarities between Parkinson's disease (PD) and manganese toxicity, it is likely that many of the key molecular players in both disorders interact in similar molecular pathways. The establishment of our *C. elegans* PD model will allow us to examine the role that DA, its metabolites, endogenous proteins and neurotoxins play in Mn induced cell death.

Body

STUDY 1 – Determine whether Mn^{2+}-induced DA neuron degeneration is dependent on DA or DA neuron-specific proteins, and the neurodegeneration can be amplified by exposure to 6-OHDA or expression of human α-synuclein animals in vivo.

A goal of our first aim is to determine the role that dopamine or dopamine-associated proteins play in Mn^{2+}- or toxin-induced cell death. Our studies have shown that the DA neurons are sensitive to Mn^{2+}, and the sensitivity is amplified following exposure to 6-OHDA. We have also shown that the vesicular monoamine transporter (VMAT) plays a role in 6-OHDA sensitivity. Our more recent studies suggest that tyrosine hydroxylase (TH) expression effect the sensitivity of the DA neurons to 6-OHDA, providing further evidence that DA level can modulate neurodegeneration and may play a role in Mn^{2+}-induced cell death. Interestingly, the TH knockout worm's sensitivity to the toxin is appears to be dependent on the age of the animals. The younger animals are much more sensitive to 6-OHDA relative to WT, while the older animals are more resistant. Although there could be other explanations, is it possible that the worm could be producing DA via another TH gene? Although not reported in literature, there is a putative overlapping gene that has potential to express TH in the worm. This is consistent with an earlier report that the DA (TH) knockout worm still produces some DA (Wintle, 2001). Although in our hands we do not find DA in the TH knockout animal as analyzed by HPLC, it is important to determine if another TH is still expressed in the knockout animal, and if so, if it is involved in the generation of DA. If so, each TH may have age dependent expression, and independently affect DA neuron degeneration vulnerability. These results could also help explain the role of DAT and VMAT, in which in mammalians systems have been shown to be modulated by DA level, and correlate with DA neuron degeneration. We have therefore designed and are currently generating antibodies to the *C. elegans* TH, DAT, VMAT, and other DA and DAT-associated proteins and these should greatly assist us in dissecting the role these proteins play in Mn^{2+}-induced DA neurodegeneration.

In vitro studies show that Mn^{2+} causes an increase in α-synuclein-induced cell death. Although the function of α-synuclein is not clear, it may play a role in synaptic vesicle binding and neurogenesis. To further explore the mechanism of α-synuclein induced cell death, we exposed α-synuclein expressing animals to 6-OHDA. We were very surprised to find that there was not an increase in cell death. Recent studies suggest that α-synuclein could play a role in *neuroprotection*. Our results are consistent with these latest findings and we now have developed a genetic tool to explore the basis for the protection. To gain further insight into the role α-synuclein may play in the cell, we performed whole genome microarray analysis in the transgenic *C. elegans*. We found changes in proteosome and mitochondrial gene expression,

consistent with vertebrate studies. These studies further supports that *C. elegans* can be a powerful model for α-synuclein induced cell death, and the work was published in 2006.

STUDY 2 – Establish and evaluate transgenic lines overexpressing edongenous parkin and normal and mutant parkin and determine whether these genes play a role in Mn^{2+}-induced neurodegeneration in both WT and cell death pathway deficient mutants.

A major goal of our second aim is to determine the role of parkin in Mn^{2+}-induced DA neuron cell death in C. elegans. Parkin has previously been shown to attenuate Mn^{2+} induced DA neuron cell death in other systems. We have previously shown that strains containing the deletion of the *C. elegans* parkin protein increases DA neuron sensitivity to 6-OHDA. We have now generated several sizes of GFP-tagged transcriptional fusions of the *C. elegans* parkin gene. Also we have almost completed the generation of several different sized translational fusions. Furthermore, we have designed and are now generating antibodies to the *C. elegans* parkin homologue. We will begin to generate transgenic animals with the transcriptional and translational fusions within a few weeks both within WT and worms deficient in cell death pathway genes. These studies should greatly assist us in determining the role that parkin may play in Mn^{2+}-induced neurodegeneration.

Key Research Accomplishments

- *C. elegans* strains with deletions in TH or VMAT were crossed animals expressing GFP in DA neuron, and examined for sensitivity to 6-OHDA and Mn^{2+}.
- Whole animal and DA neruon sensitivity to Mn^{2+} was examined as a function of both a time and concentration.
- The DA neurons are sensitive to RNAi
- *C. elegans* animals expressing α-synuclein and parkin deletion lines were examined for Mn sensitivity.
- Microarray studies were performed using Affymetrix Arrays.
- Several transcriptional and translation GFP fusions to the Parkin gene were generated using standard molecular biology techniques
- Antibodies to TH, VMAT, DAT, parkin, and other heavy metal-associated proteins are being generated using a novel method to significantly increase antigenicity.

Reportable Outcomes

Vartiainen, S., Pehkonen, P., Lakso, M., **Nass, R**., Wong, G. (2006) Identification of gene expression changes in transgenic *C. elegans* overexpressing human α-synuclein. *Neurobio. Dis*. **22**:477-486

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Nass, R., Hamza, I. (2007) The nematode *C. elegans* as a model to explore toxicology in vivo: solid and axenic growth culture conditions and compound exposure parameters, *Curr Protocols Toxicology,* 1.9.1-1.9.18

Nass, R., Chen, L. (2007) *C. elegans* models of human neurodegenerative diseases: a powerful tool to identify molecular mechanisms and novel therapeutic targets. In: Sourcebook of Model Organisms in Biomedical Research, ed. P.M. Conn. Humana Press. *Manuscript in press.*

Nass, R. and Przedborski, S., "Parkinson's disease: pathogenic and therapeutic insights from toxin and genetic models," Reference Book, Elsevier Press. Expected release date: Summer 2008

Conclusions

We have now shown that DA levels that are modulated by TH and VMAT may affect DA neuron sensitivity to neurotoxins, and this affect may be age dependent. We have generated transcriptional and translation GFP fusions to a number of Mn^{2+}-associated and PD associated homologues in the worm. Finally, we are generating antibodies to these same proteins, as well as others that have been implicated in PD and heavy metal-induced toxicity. Our recent α-synuclein studies suggest that this protein has both neurotoxicity and neuroprotective roles, consistent with recent vertebrate studies, and it is very likely that Mn^{2+}-exposures will modulate the role. These tools should greatly facilitate our studies to identify and characterize the proteins involved in DA neuron degeneration.

Current faculty receiving support from the grant:

- Richard Nass, Ph.D

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TITLE: Molecular Mechanisms Underlying Mn Neurotoxicity

PRINCIPAL INVESTIGATOR: BethAnn McLaughlin, PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203

REPORT DATE: January 2008

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Fort Detrick, Maryland 21702-5012

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14. ABSTRACT

Accumulating evidence supports that cell death associated with a host of neurological disorders including Alzheimer's and Parkinson's diseases (AD and PD respectively) is potentiated by exposure to manganese (Mn). The gene-environment interactions are particular significant given the association of abnormally high brain Mn levels with the PD symptoms, and that occupational exposure to Mn is common especially in welders. Inspite of compelling epidemiological data suggesting that welders may be at high risk of undergoing cell death thru occupational exposure, little is known about molecular mechanisms underlying Mn neurotoxicity. The goal of our 2-year study is to begin to understand molecular mechanisms underlying Mn neurotoxicity using a combination of chronic in vivo and in vitro models; we detailed the damages caused by Mn exposure in cultures and identify molecular signaling pathways which mediated degeneration. We found early changes in cytoskeletal structure associated with AD-type pathology, that these changes were more strongly associated with dopaminergic neurons than other types of neurons and that there is profound circuit level compensation for loss of dopaminergic innervation when animals were chronically exposed to Mn. Taken together, these data suggest that manganese is a potent neurotoxin to dopaminergic cells, that the primary sites of initial degeneration are in distal cytoskeleton and that even small chronic levels of manganese (5 mg/kg/day) were capable of killing 20% of doapminergic cells in the substantia nigra and resulting dysfunction in the striatum and globus pallidus.

15. SUBJECT TERMS

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Introduction

Manganese (Mn) is an essential nutrient, integral to proper metabolism of amino acids, proteins, lipids, and carbohydrates. While Mn plays roles in normal cell functioning, high levels of Mn are neurotoxic. This problem is particularly relevant in the context of occupational exposure to Mn by miners and welders which has been linked to the onset of a neurological phenotype, known as manganism, which presents with motor symptoms resembling those of Parkinson's disease (Aschner and Aschner 1991; Lee 2000; Dobson, Erikson et al. 2004; Erikson, Jones et al. 2005; Santamaria, Cushing et al. 2007).

Consistent and deleterious behavioral consequences of chronic elevated manganese exposure have been demonstrated in a variety of animal models (Normandin, Ann Beaupre et al. 2004; Dodd, Ward et al. 2005; Torrente, Colomina et al. 2005; Guilarte, Chen et al. 2006; Liu, Sullivan et al. 2006). The alterations that have been observed are reminiscent of clinical manganism, and include effects on motor control, dystonia, and neuropsychiatric abnormalities. At the cellular level, however, the specificity of Mn at targeting cells killed by PD is less clear. A wide variety of alterations have been observed with Mn exposure it has not yet been determined if neurotoxin Mn specifically targets dopaminergic cells killed in PD or how neural cells respond to Mn exposure. **The purpose of this work was to determine if chronic exposure to Mn results in selective dopaminergic degeneration and if dopaminergic neurons are uniquely and/or inherently vulnerable to Mn.**

Body

STUDY 1 – *In Vitro* Modeling of Manganese Toxicity

Dopaminergic neurons of the substantia nigra are a highly vulnerable to oxidative, excitotoxic and metabolic stressors, and while the clinical manifestations of Mn exposure parallel PD, it is unclear if dopaminergic neurons are inherently and uniquely vulnerable to Mn (Santamaria, Cushing et al. 2007). To address this question, we used primary cultures from mesencephalon to determine the extent of cell death induced by Mn. Having established dose response relationships of Mn and survival, we next treated cells with minimally toxic levels of Mn to determine if the dopaminergic populations in these cultures underwent more dramatic cytoarchitectural changes than other populations.

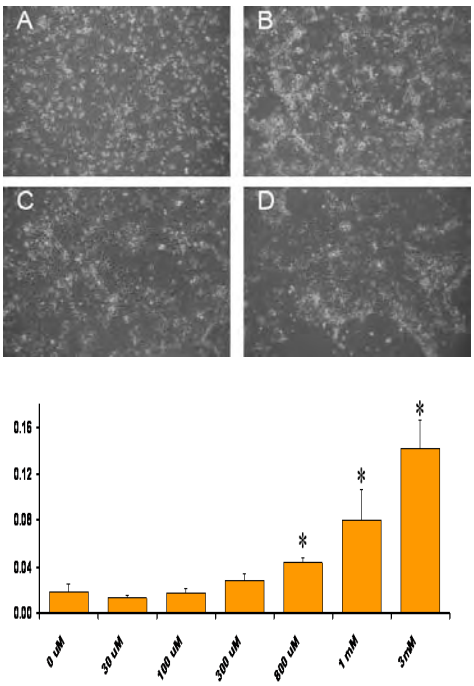

Figure 1: *Manganese Exposure Induces Neuronal Death in Mesencephalic Cultures. In vitro* exposure to $MnCl_2$ for 24 hr inuces appreciable cell death in primary cultures as assessed by LDH release. Compared to (A) control, disruption of neuronal processes and excess cellular debris are visible in representative images from (B) 1 mM $MnCl_2$, (C) 3 mM $MnCl_2$, and (D) 10 mM H_2O_2 treatments. Quantification of cell counts revealed significant cell death at exposures of 800 uM for greater than 24h. * $p < 0.05$.

Manganese is a Potent Neurotoxin Which Causes Rapid and Irreversible Cell Death in Culture. Primary neuron enriched cultures were exposed to increasing concentrations of Mn for

24h. These mesenchephalic cultures were grown for 14-18 days in vitro to promote neurite outgrowth and maturation using our previously described method (McLaughlin, Nelson et al. 1998). Cell viability was assessed 24h later using lactate dehydrogenase release from dead and dying neurons (Aizenman, Stout et al. 2000). We observed that Mn induced cell death at > 800 micromolar exposures (Figure 1).

Cytoskeletal Changes in Dopaminergic Neurons Occur Rapidly and with Relatively Low Level Exposure

Using subtoxic levels of Mn from the studies above, we next sought to determine the pathological hallmarks of Mn toxicity using immunohistochemistry. Neural architecture is coordinated by an elaborate series of structural and trafficking proteins that rely upon a microtubule infrastructure. These proteins play obligatory functions in the maintenance of cellular transport, and alterations in tubulin protein expression or posttranslational modification state by toxins cause significant damage to the cell; these concerns are particularly pertinent when considering the fate of long projection neurons, such as dopaminergic neurons in the nigrostriatal pathway (Ren et al., 2003; Callio et al., 2005). Both α- and β-tubulins have been implicated in PD pathology (Landino, Robinson et al. 2004; Diaz-Corrales, Asanuma et al. 2005; Yang, Jiang et al. 2005; Feng 2006; Chen, Jin et al. 2007). We chose to evaluate tubulins as well as the microtubule-associated protein tau. Tau plays an essential role in the stabilization of the microtubule tracks and mutations in tau have been linked to both AD and PD pathology. The hyperphosphorylation of tau-1 by proline-directed kinases and self-aggregation occur are a hallmark feature of AD and as well (Ballatore, Lee et al. 2007). We observed significant changes in cytoskeleton, in neurites and synapses with low-level chronic Mn exposure in vitro (Figure 2). Mn treatment causes neurite fragmentation with aggregation of tau, beta-tubulin and alpha-synuclein proteins, and eventually apoptotic cell death of mesenchephalic neurons. Even at a low concentration before eliciting cell death, Mn treatment caused appreciable changes in tau distribution, suggesting that cytoskeletal infrastructure derangement is an early event in Mn induced neurotoxicity.

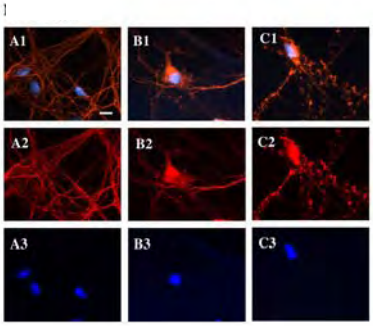

Figure 2 Minimally Toxic Exposure to Manganese Results in Distal Neurite Fragmentation and Loss of Tau Immunostaining in Mesencephalic Neurons. A1-A3 – control; B1-B3 – 100 μM manganese; C1 –C3– 800 μM manganese; cells were continuously exposed to toxin for 24h and fixed for immunohistochemistry. Panels A2-C2 show tau-1 (red) immunostaining and panels A3-C3 show bis-benzimide (blue) labeled nuclei.

Conclusions

While Mn induces toxicity in mesenchephalic neurons, it is not known if Mn *selectively* targets these cells or the mechanism by which cells die are similar to PD induced degeneration. Our data demonstrates that Mn is profoundly neurotoxic and that even at minimally toxic exposures, neurites are particularly vulnerable to Mn exposure. While beyond the work in this two year grant, we believe that our results in combination with those of Dr. Aschner presents a larger picture of cell stress consistent with PD pathology. That is, neurite breakdown and tau dysfunction are common effects of PD and AD mutations and associated with oxidative stress. Dr. Aschner's work as a part of the MHRP suggests that oxidative stress is a significant component of Mn injury. There is also compelling evidence to suggest that dopaminergic

neurons die preferentially when exposed to oxidant stress and that oxidant stress contributes to PD pathology (Zhang, Dawson et al. 2000; Blum, Torch et al. 2001).

STUDY 2 – *In Vivo* Modeling of Manganese Toxicity Reveals Appreciable Degeneration of Dopaminergic Regions and Neurochemical Alterations in Projection Areas Similar to PD.

The clinical manifestations of Parkinson's disease (PD) result from the degeneration of mesencephalic dopaminergic neurons and the associated decrease of striatal dopamine content. According to current models of PD pathophysiology, the lack of stimulation of striatal dopamine receptors will produce specific functional changes in neurons of the basal ganglia direct and indirect pathways. Specifically, loss of dopaminergic projections to the striatum striatal has been associated with a regionally selective increase in striatal nerve-ending GABA synthetic capacity which is rate limiting by the enzyme GAD.

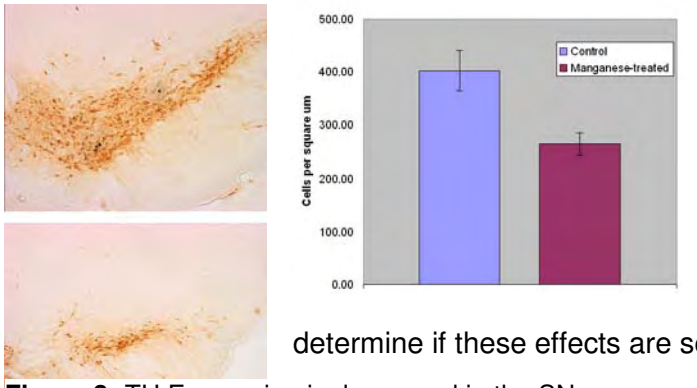

Figure 3: TH Expression is decreased in the SNpc following chronic Mn treatment. Immunohistochemical analysis followed by cell counts demonstrated a significant decrease in number of TH-expressing neurons within the SNpc of Mn-treated mice compared to controls. Representative images from the SNpc of a control mouse (top) and Mn-treated mouse (bottom). Total cell number is significantly reduced in the Substantia Nigra following chronic Mn exposure. Immunohistochemical analysis showed a significant decrease in cresyl-violet stained celll bodies throughout the SNpr and SN pc. Asterisks represents $p<0.05$.

In our *in vitro* work, we determined that mesenchephalic cultures were highly vulnerable to Mn and that changes in neural structure were an early event in degeneration. In order to determine if these effects are selective and specific to dopamine cells when Mn is delivered peripherally and chronically, we next moved to an animal model of manganese-induced stress.

Mice were given chronic IP injections of Mn for 30 days. Using immunohistochemical analyses and cell counting, we determined there was a dramatic decrease in the expression of tyrosine hydroxylase, an enzyme required for the production of dopamine, in the substantia nigra of Mn-treated animals compared to controls.

Immunohistochemical stains for GAD67, the rate-limiting enzyme in GABA production were undertaken throughout the basal ganglia.

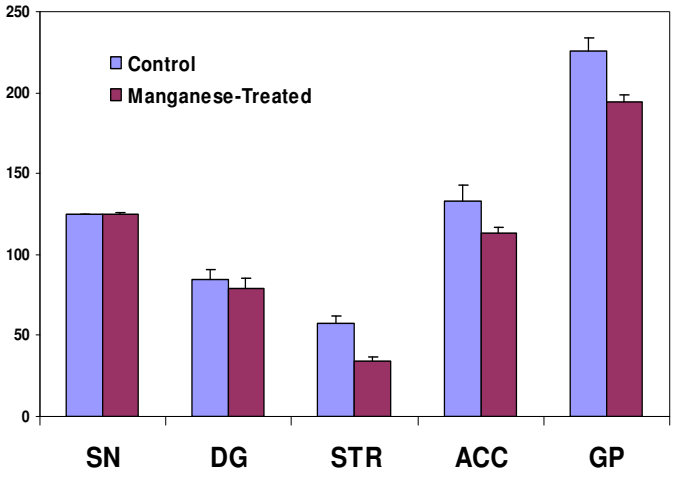

Figure 4: GAD67 expression is decreased in the CPu and GP following chronic Mn treatment. Immunohistochemical analysis followed by cell counts demonstrated significant decreases in the numbers of GAD67 expressing neurons within the CPu and the GP of Mn-treated mice. This decrease was 39.4% in the CPu and 14% in the GP. Representative images of the CPu (A,B) reflect a decrease in the number of GAD67+ interneurons. Images (C) and (D) show decreased expression in the GP. Asterisks represent $p<0.05$.

Conclusion: The results of these studies suggest that Mn exposure can produce neurochemical dysfunction in key areas of the basal ganglia, including the striatum and globus pallidus. These data suggest that far from being contained to on subset of neurons, chronic Mn exposure ultimately has long lasting and region specific effects, which are reminiscent of PD pathology.

Key Research Accomplishments

- Mn-exposure can induce changes in protein profiles throughout basal ganglia circuitry, interacting with both the direct and indirect pathways.
- Tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, shows decreased expression through SN, consistent with PD pathology and clinically similar manganism.
- GAD67, the rate-limiting enzyme in GABA synthesis, shows decreased expression in both STR and GP areas but is unaltered in the SN.

Conclusion

The conclusions from the studies described above are consistent with Mn's ability to cause profound neurotoxicity even at minimally toxic exposures. Neurites are particularly vulnerable to Mn exposure. There is also compelling evidence to suggest that dopaminergic neurons die preferentially when exposed to oxidant stress and that oxidant stress contributes to PD pathology. Our results are also consistent with the ability of Mn to produce neurochemical dysfunction in key areas of the basal ganglia, including the striatum and globus pallidus. These data suggest that far from being contained to on subset of neurons, chronic Mn exposure ultimately has long lasting and region specific effects, which are reminiscent of PD pathology.

Manuscripts in Preparation:

Stanwood, G.D., Leitch, D.B., Anderson, D.J., Fitsanakis, V.A., Aschner, M and McLaughlin, B.A. *Chronic Manganese Exposure in Mice is Neurotoxic and Alters Dopaminergic and GABAergic Neurons within the Basal Ganglia.*

Savchenko, V., Leitch, D.B., Stanwood, G.D., Aschner, M., Wu, J. and McLaughlin, B.A. *Manganese Induces Cytoskeletal Rearrangement and Apoptotic Death of Dopaminergic Neurons.*

Current faculty receiving support from the grant:

BethAnn McLaughlin, PhD

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TITLE: Exposure to Welding Fume and Parkinson's disease: a feasibility study.

PRINCIPAL INVESTIGATOR: Prof Craig Jackson and Prof Jouni Jaakkola

CONTRACTING ORGANIZATION: Institute of Occupational and Environmental Medicine, University of Birmingham, Edgbaston, Birmingham UK. B15 2TT

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14. ABSTRACT

A case-control study of a small group of welders with typical idiopathic PD (Racette et al. 2001) showed no clinical differences between them and the typical PD population, but their disease was distinguished by a statistically significant younger age of onset (46 years) for welders compared with 63 years for the controls. This promoted the hypothesis that employment as a welder may be a risk factor for PD, accelerating or triggering the onset of the disease. This remains controversial but impossible to dismiss. A case-referent study represents the most appropriate means of investigating this theory. The objectives of the study are to test the following hypotheses:
I. A significantly higher proportion of those diagnosed as having PD have been welders of steel, or otherwise exposed to manganese-containing metal fumes, than those in a matched control group (age and sex) who do not have that diagnosis.
II. Within those diagnosed with PD, the age of onset is lower among those who have been occupationally exposed to manganese than those who have not.
Federal Wide assurance has been obtained by the researchers and ethical approval for the study has been granted both locally (Solihull LREC) and by the USAMRMC. Twice-weekly data collection has now commenced with PD patients in 2 hospital clinics, with control-population data collection to commence soon, and this is due to continue for the next 6 months.

15. SUBJECT TERMS
Manganese. Exposure. Steel Welding. Parkinson's Disease. Manganism.

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Introduction

Parkinson's disease (PD) is a common neurological disorder affecting one in 500 of the general population and 1% of those over the age of 65. The first observable symptom of Parkinson's disease is often tremor (trembling or shaking) of a limb, especially when the body is at rest. The tremor is initially unilateral, frequently in one hand. Other common symptoms include slow movement (bradykinesia), an inability to move (akinesia), rigid limbs, a shuffling gait, and a stooped posture. People with Parkinson's disease often show reduced facial expressions and may speak quietly. Occasionally, the disease also causes depression, personality changes, dementia, sleep disturbances, speech impairments, or sexual difficulties. The severity of Parkinson's symptoms tends to worsen over time. There is no known causal agent, although undefined environmental factors are suspected, possibly through interactions with genetic susceptibility. This has led to the identification of idiopathic Parkinson's disease, although other types of PD exist with better understood causes, although this only accounts for as small number of cases, and these are brought together under the term Parkinsonism. Research currently indicates that genetic factors are most likely to predispose patients to develop PD if combined with other gene mutations or environmental toxins.

A similar collection of symptoms to those displayed in idiopathic PD is a specific neurological disorder that may be attributed to excess absorption of manganese is manganism, (known as chronic manganese intoxication) typified by slowly progressive deterioration of well-being coupled with specific disturbances of mood and muscle function. Manganese is recommended in dietary intake (2 to 5 mg/day), and manganese is included in parenteral nutrition. The toxic effects in manganism are considered to result from interference by manganese in the metabolism of biogenic amines such as dopamine, and *in vitro* welding fume enhances dopamine oxidation, raising the possibility that manganese in welding fume may contribute to dopamine deficiencies in those who develop PD. Manganese-induced neurotoxicity has been reported to occur only after chronic exposure to high levels of manganese, usually above the permissible exposure limits. The disorder bears a marked similarity to Parkinson's disease (PD) and has been referred to as manganese-induced Parkinsonism or secondary Parkinsonism. Exposure to high airborne levels of manganese in foundry and mine workers has historically been associated with a neurotoxicity that resembles Parkinson's disease.

Today, the greatest use of manganese ores is in the production of iron, steel, and manganese alloys, and when welding such metals and flame cutting, fumes that contain several respirable substances including manganese are produced. If the suggested link between manganese exposure and PD-like symptoms is correct, it would be expected that that low grade exposure to manganese fumes (commonly experienced by metal welders) may increase the risk for the development of Parkinson's disease and other basal ganglia and movement disorders in some workers.

It has also been proposed that compounds released during welding and flame cutting may accelerate the onset of Parkinson's disease. However, the number of well designed epidemiological studies and cohort studies which have evaluated these occupational issues is small, however, in part because Parkinson's Disease is relatively rare and most often occurs in older age groups. While the link between manganese and the development of PD remains controversial it is currently impossible to dismiss the possibility that occupational exposure to manganese compounds in welding fume may enhance genetic susceptibility to or interact with other environmental agents that may cause or expedite the onset of PD. A larger-scale study may be required.

Manganese oxides are a constituent of the fume emitted from cutting and arc welding of steel and applying hard facing to tools. Steel welders probably constitute the largest occupational group exposed to manganese and its compounds. Some research has suggested that welders may be at a higher risk for developing Parkinsonism, and some have proposed that welding is a risk factor for PD. Such claims have entered the legal system and lawsuits have been filed on behalf of welders alleging that toxic fumes generated by welding rods (containing manganese) have caused not just Parkinsonism, but also Parkinson's disease. However, several mortality surveys of large populations of welders found elevated rates of certain cancers, accidents strokes, cirrhosis, heart disease and even suicide, but not of PD or any

other neurological diseases (Coggon *et al.* 1995). Conversely, other research has shown welders to have lower levels of PD than control occupations (Kirkey *et al.* 2001).

Body

A case-control study of a small group of welders with typical idiopathic PD (Racette *et al.* 2001) showed there were no clinical differences between them and the typical PD population, but their disease was solely distinguished by the younger age of onset for welders (46 years) compared with the control group (63 years). Despite the significant and important results of the Racette *et al.* study, the study did not provide any evidence that welders were more likely to develop PD than the general population; the authors suggested that welding acted to accelerate the onset of PD. There were some important methodological limitations to the study that must be considered, mostly concerning the appropriateness of the welders involved: especially as patients were not randomly selected and there was no evidence that the welders in the study were representative of all welders. The data was collected from a center specializing in Parkinsonism disorders, (rather than a general movement disorder clinic) which may have raised the possibility of referral bias, as patients referred to specialty clinics may often be more atypical than general PD patients. Additionally, the method of referral to the specialty clinic was not specified, and 53% of the welders in the study had a family history of PD - higher than the 15% reported in unselected PD patients. Importantly there is a known link between family-history and younger-onset PD, possibly complicated by genetic factors. It has been concluded that genetics rather than welding was the major risk factor in these relatively young patients with PD who happened to be welders (Jankovic 2005).

Given the contradictory nature of the research body, it is proposed that a case-referent study represents the most appropriate means of investigating this hypothesis. The feasibility study would involve the identification of a population of males diagnosed with PD and a population of controls matched for age, gender and socio-economic status. The study population will be restricted to males only, since the majority of welders are male. Detailed assessment of work, clinical and smoking history would be carried out for each case and control. The work history would focus particularly on work as a welder and exposure to metal fumes, in order to provide an estimate of the extent and nature of this exposure (please see Figure 1). The objectives of such a study would be to test the following two hypotheses:

H_i 1:
A significantly higher proportion of those diagnosed as having PD have been electric arc welders of steel, or otherwise exposed to manganese - containing metal fumes, than those in a matched control group (age, sex, education) who do not have that diagnosis.

H_i 2:
Within those diagnosed as having PD, the age of onset will be lower among those who have been occupationally exposed to manganese than those who have not.

Aims:
Primary.
If any manganese-effects are detected or nearly detected, the aim of this study is to suggest the methodological and statistical feasibility of a larger-scale study.

Secondary.
To identify if occupational exposure to manganese (mainly derived from welding of steel) is associated with the development of Parkinson's Disease among males, in terms of a younger age of onset among exposed PD patients than non-exposed PD patients. The age of onset of PD may also be related to time as a welder and the estimated dose of welding fumes and metal fumes.

Key Research Accomplishments to date

a) Applied for and gained Local Research Ethical Committee approval to conduct a feasibility study.

b) Secured Federal Wide assurance (to facilitate USAMRMC ethical approval)

c) Applied for and gained USAMRMC approval to conduct a feasibility study.

d) Developed patient / control participant (i) info sheets (ii) consent forms (iii) data collection sheets (iv) occupational history questionnaires

e) Secured access to clinical population at two movement disorder clinics and a suitable matched-control group of non-PD healthy volunteers.

f) Commencement of data collection from PD patients in the two movement disorders clinics commenced in January 2008 (for approximately 6 months).

g) Commencement of data collection from Control volunteers will commence in March 2008 (for approximately 4 months).

Conclusions

This study is in its early stages and it would be premature to derive any conclusions prior to additional data collection and analysis. The conclusions will be reported in the 09 Progress report.

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14. ABSTRACT

Neurotoxicity is a significant occupational and public health concern linked with high-dose manganese (Mn) inhalation. We used liquid chromatography-mass spectrometry (LC-MS) methods in the identification of blood and urine biomarkers from rhesus monkeys following subchronic air or Mn sulfate (MnSO4) inhalation. Juvenile rhesus monkeys were exposed 5 days/week to airborne $MnSO_4$ at 0, 0.06, 0.3 or 1.5 mg Mn/m^3 for 65 exposure days or 1.5 mg Mn/m^3 for 15 or 33 days. Monkeys exposed to $MnSO_4$ at • 0.06 mg Mn/m^3 developed increased Mn concentrations in the globus pallidus, putamen, olfactory epithelium, olfactory bulb, and cerebellum. Statistically significant changes in serum metabolic profiles were observed from Mn-exposed monkeys. In all, 27 metabolites with statistically significant expression differences were structurally confirmed by MS-MS methods. Biochemical changes identified in Mn-exposed monkeys included endpoints related to oxidative stress (e.g., oxidized glutathione) and neurotransmission (aminobutyrate, glutamine, phenylalanine).

15. SUBJECT TERMS
Manganese, neurotoxicology, metabolomics, welding, manganese mining

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Introduction

Manganese neurotoxicity is a significant public health concern associated with welding, metal smelting operations, steel production, and foundries (Levy and Nassetta, 2003). Under conditions of high occupational exposure, excess manganese accumulates within the human striatum and globus pallidus and produces damage to dopaminergic neurons within these sites (Malecki et al., 1999; Nelson et al., 1993; Pal et al., 1999). Early manifestations of manganese neurotoxicity include fatigue, headache, muscle cramps, loss of appetite, apathy, insomnia, and diminished libido. As exposure continues and the disease progresses, patients may develop prolonged muscle contractions (dystonia), decreased muscle movement (hypokinesia), rigidity, and muscle tremors (reviewed by Pal et al., 1999).

Manganese toxicity may occur following high dose ingestion or parenteral injection (Calne et al., 1999; Nagatomo et al., 1999). For example, Kawamura and coworkers (1941) and Kondakis et al. (1989) documented outbreaks of manganese toxicity in Japan and Greece due to the consumption of water from wells contaminated with extremely high levels of manganese (1.8 to 14 mg Mn/L). Neurotoxicity following manganese exposure, however, is most frequently observed following inhalation of this metal. Brain delivery of manganese is higher following inhalation versus ingestion, and pharmacokinetic factors that contribute to this increased efficiency in brain manganese delivery include increased manganese absorption from the pulmonary tract, slower blood clearance of absorbed manganese, and direct delivery to the brain via the olfactory system (Andersen et al., 1999; Aschner et al., 2005). Occupational manganese neurotoxicity most commonly occurs in individuals that have been chronically exposed to aerosols or dusts that contain extremely high levels (> 1 mg Mn/m^3) of manganese (ATSDR, 1992; Mergler et al., 1994; Pal et al., 1999). There is a growing concern that welders and other steel workers may be at increased risk for manganese neurotoxicity (Kim et al., 1999; Lucchini et al., 1999; McMillan, 2005).

Identification of workers with excess manganese exposure primarily relies on workplace exposure assessment studies and/or measurement of appropriate biomarkers of exposure. Biomarkers are biological parameters that can be used to identify a physiological or pathological state. There are few biomarkers that correlate reliably with excess manganese exposure (Aschner et al., 2005; Greger, 1999). Brain magnetic resonance imaging (MRI) holds promise as a biomarker of manganese exposure (Kim, 2004). Because manganese is paramagnetic, its relative distribution within the brain can be examined using MRI. Neuroimaging studies in humans have documented that either increased manganese exposure, or reduced hepatobiliary excretion of manganese, can result in appreciable MRI hyperintensities within the pallidum and other brain regions known to accumulate manganese (Ikeda et al., 2000; Kim, 2004; Krieger et al., 1995; Nagatomo et al., 1999; Rose et al., 1999; Sadek et al., 2003; Stewart et al., 2005). Brain MRI has been used to support a diagnosis of manganese neurotoxicity in welders, individuals receiving total parenteral nutrition, and patients with hepatobiliary insufficiency (Kim, 2004; Nagatomo et al., 1999; Stewart et al., 2005). Brain MRI evaluations also hold the potential to provide semi-quantitative estimates of brain manganese concentrations (Dorman et al., 2006b). Despite these advancements, the expense and effort associated with MRI approaches may restrict its use to focused clinical or epidemiological research studies.

There are mixed reports as to whether urinary manganese excretion is increased following occupational inhalation exposure (Ellingsen et al., 2005; Ellingsen et al., 2006; Lu et al., 2006). This finding likely reflects low urinary excretion of this metal (< 1% of the absorbed dose). Blood, serum, and plasma manganese concentrations depend upon the magnitude and the duration of the manganese exposure and often demonstrate only a weak association with workplace manganese exposure concentrations (Aschner et al., 2005; Ellingsen et al., 2003; Ellingsen et al., 2006; Lu et al., 2006). Measurements of arginase and lymphocytic manganese-superoxide dismutase activity have been touted as potential biomarkers of exposure; however, these endpoints have failed to gain broad acceptance among toxicologists (Aschner et al., 2005). The identification of suitable biomarkers of manganese exposure has seen few other recent advances.

High throughput analytical chemistry approaches provide one means of identifying novel biomarkers. ‘Metabolomics’, the metabolite analog of genomics and proteomics, is the global analysis of all metabolites in a sample (or "metabolite profiling") (Raamsdonk et al., 2001; Weckwerth and Morgenthal, 2005), while ‘metabonomics’ represents the analysis of metabolic responses to a xenobiotic or disease state. In metabolomic research, analytes of interest are separated by their chemical properties (e.g. hydrophobicity, hydrophilicity, or charge). Developments in high performance liquid chromatography (HPLC), gas

chromatography, and capillary electrophoresis when coupled with mass spectrometry (MS), i.e., HPLC–MS, GC–MS and CE–MS, have greatly contributed to recent advancements in this field. Metabolomics has been used to identify biomarkers associated with renal cancer (Perroud et al., 2006), amyotrophic lateral sclerosis (Bowser et al., 2006), myocardial ischemia (Sabatine et al., 2005), and other disease states (Oresic et al., 2006). Metabolic profiling has also been applied to toxicology with recent applications to cisplatin-induced nephrotoxicity (Portilla et al., 2006) and other types of metal toxicities (Fowler at al., 2005). These studies have clearly demonstrated the potential of metabolomics, often coupled with proteomics, to identify new biomarkers of exposure or disease.

The goal of this study was to search for novel biomarkers of manganese exposure in biological fluids obtained from rhesus monkeys following high-dose manganese sulfate ($MnSO_4$) inhalation. Completion of this project relied on tissue samples archived from a subchronic $MnSO_4$ inhalation study performed in rhesus monkeys.

Body

Animals: This study was conducted under federal guidelines for the care and use of laboratory animals (NRC, 1996) and was approved by the CIIT Institutional Animal Care and Use Committee. Twenty-eight male rhesus monkeys purchased from Covance Research Products, Inc. (Alice, TX) were used in this study. Monkeys were 17 to 22 months old at the time of their arrival at CIIT. Monkeys were approximately 20 to 24 months old at the start of the inhalation exposure. Twenty monkeys were exposed 6 hr/day, 5 days/wk, for 13 wk (65 exposure days). These monkeys were allocated as follows: air (n = 6), 0.06 (n = 6), 0.3 (n = 4), and 1.5 (n = 4) mg Mn/m^3. The remaining monkeys were exposed to $MnSO_4$ at 1.5 mg Mn/m^3 for 15 (n = 4) or 33 (n = 4) exposure days and evaluated immediately thereafter. Additional details concerning these animals and their husbandry has been published (Dorman et al., 2005).

Manganese exposures: Manganese (II) sulfate monohydrate (MnSO4•H2O) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Four 8-m3 stainless steel and glass inhalation exposure chambers were used. Methods describing chamber monitoring as well as generation and characterization of the MnSO4 aerosol has been previously described (Dorman et al., 2004; 2005). Based upon optical particle sensor measurements, the overall average concentrations (± SD) for the $MnSO_4$ atmospheres were 0.19 ± 0.01, 0.97 ± 0.06, and 4.55 ± 0.33 mg/m^3 for the target concentrations of 0.18, 0.92, and 4.62 mg $MnSO_4/m^3$, respectively. The geometric mean diameter, geometric standard deviation (σ_g), and calculated mass median aerodynamic diameters (MMAD) of the $MnSO_4$ aerosols were determined to be 1.04 µm (σ_g = 1.51; MMAD = 1.73 µm), 1.07 µm (σ_g = 1.54; MMAD = 1.89 µm), and 1.12 µm (σ_g = 1.58; MMAD = 2.12 µm) for the target concentrations of 0.18, 0.92, and 4.62 mg $MnSO_4/m^3$, respectively.

Metabolomic profiling and pathway analysis: Metabolomic analyses were performed at Icoria, Inc. (Morrisville, NC) and included liquid chromatography-mass spectrometry (LC-MS) measurement of a broad and non-selective range of low molecular weight (< 1,000 amu) biochemicals including metabolites and small peptides in the urine and blood from the $MnSO_4$-exposed monkeys. Specifically, liquid chromatography time of-flight mass spectrometry (LC-TOF-MS) was used for chromatographic separation of metabolites, followed by detection by mass. The LC-MS platform consists of Bruker time of flight (TOF) instruments coupled to an internally developed HPLC method. These TOF instruments offered high sensitivity and mass accuracy (5-10 ppm depending on molecular mass). Measuring metabolites in simultaneous ± ESI modes enabled the identification of more metabolites than either mode alone could achieve.

Blood and urine samples were prepared for metabolomic analysis by thawing, and precipitating proteins, followed by acetonitrile extraction of the metabolites. Serum was deproteinized with aqueous acetonitrile, the precipitate spun down, and the extract dried under vacuum. The residue was reconstituted with aqueous acetonitrile. Urine was diluted with aqueous acetonitrile, and cleared by centrifugation. A series of 6 internal standards were added to the extracts for QC purposes. Samples were prepared in triplicate, randomized, and analyzed in a 96 well plate format. Up to sixteen quality control standards plus blanks were plated within the 96 well plates.

Typically, 10 µL of samples were injected into an Agilent 1100 series HPLC system. LC separations were performed on an Atlantis® dC_{18}, 3 µm, 2.1 × 100 mm column (Waters, MA, USA) equilibrated with solvent A, which consists of H_2O containing 5 mM ammonium acetate (pH 5.5). The components were eluted at a flow

rate of 0.25 mL/min with a gradient elution of 0-0.2 min: 0-0% B (CH_3CN); 0.2-8 min: 0-50% B; 8-9 min: 50-50% B; 9-10 min: 50-75% B.

LC-MS peaks from each sample were aligned by mass to charge (m/z) ratio and retention time (RT) across all samples for each matrix, and quantified using R/XCMS. LC-MS components for each replicate of each sample are represented mathematically as a vector. Each component in each sample had three associated measurements: raw intensity in each sample; chromatographic RT for peaks; and the mass divided by the charge (M/z). The metabolomic profile of each sample (denoted as x) was defined by the set of all components of known intensity, RT, and mass divided by charge. Before analyzing the data we conducted the following preprocessing steps:

- Normalization to internal standard: Each metabolomic profile, x, was normalized using the intensity of a standard compound ('internal standard', which was added to each sample), transforming x into a relative intensity profile. This step was necessary to address small systematic variations of raw intensity measurements between samples due to instrument signal fluctuation. For this purpose, the internal standard need not be chemically or structurally related to the metabolite(s) of interest. Six internal standards were added to each matrix and the internal standard giving the most consistent responses (best separation in m/z axis, least matrix suppression, best peak shapes, etc.) was selected. As an example, these were usually d3-methionine for serum and d5-tryptophan for urine.
- Data quality: Assessment of technical variability: The quality of the metabolomic data was visually assessed through the distribution of the coefficient of variation (CV) for each aligned LC-MS component across technical replicates. The technical (machine) replicate variation in components was measured using the CV of the relative intensities (where $CVjk = \sigma jk/\mu jk$, where σjk = standard deviation of component k in sample j, μjk = mean of component k in sample j). The mean value of the relative intensity, μjk, for each component was used to build the average metabolomic profile for each subject. In general, median CVs were between 5-10%, and > 80% of components had a CV < 20%.
- Missing value correction: Components that were not observed across the three machine replicates were treated stringently, using deletion. If the component was observed across all replicates, μjk was calculated using three relative intensity values. When the component was observed in only two subjects, μjk was calculated between two observed values. When the component was only observed in one replicate, μjk was set to that observed value (a low intensity value).
- Distribution of relative intensity and log transformation: Though the literature on the intensity distribution of metabolomic data is limited, in our studies we have found this close to the lognormal probability density function. There were two main reasons to consider this transformation: (a) biologically, this transformation enables consideration of low concentration metabolites that capture subtle but important effects; and (b) statistically, this transformation is important for measuring the similarity between the biochemical profiles of samples (through a distance metric). Hence, we analyzed the intensity distribution in the biochemical profiles of the samples for skewness visually and transformed the data logarithmically (base e and 10) if it was lognormal.
- Data quality: clustering of technical replicates: Furthermore, the reproducibility of the metabolomic profiles was evaluated by clustering the technical (machine) replicates. We assessed the quality of replication by comparing metabolomic profiles for each subject from each tissue by using hierarchical agglomerative clustering using Pearson correlation as the distance metric. Technical replicates were found to group together consistently. A component represents a single molecule or a group of molecules with identical molecular formulae and similar physicochemical characteristics (e.g., isomers) that bin together on the m/z-retention time grid during alignment of peaks. A technical replicate as defined herein, refers to an aliquot of the same sample plated on different wells of a plate, in random fashion (as opposed to an independent extraction of the same sample).

Component annotation: Each peak group within a biochemical profile was compared to a reference in-house database of over 700 known components to assign a probable identity. This database was created by measuring the RT, m/z, and intensity for a series of standards using the LC-MS platform. Standards were selected using Icoria's proprietary database of mammalian metabolites as well as external sources of

metabolic information. For components that remain unknown or are considered to be critical members of a biomarker panel, we queried additional internal and external compound data bases (eg. Kegg, NIST, PubChem) for matches in exact mass, and then eliminated xenobiotic molecules or molecules that are not reasonable from a polarity perspective, to deduce a possible identity The list of possible matches was further refined using True Isotopic Patterns derived from the MS raw data.

Confirmation of chemical identity: Once a tentative identity was assigned, it was confirmed by fragmenting the component of interest using tandem MS (MS/MS) and comparing its fragmentation pattern to that of the known standard. The mass spectrometer used for structural identification was a Thermo Finnigan LCQ-Deca ion trap with electrospray ionization. MS conditions were as follows: sheath gas (N2 used) at a flow rate of 80% (arbitrary units); auxiliary gas at a flow rate of 20% (arbitrary units); capillary temperature, 300°C; capillary voltage, 3 V; and spray voltage, 3 kV. MS/MS experiments were carried out using helium as the collision gas. Collision energies were adjusted (typically 35% with 100% collision energy corresponding to 5 V peak to peak) in order to obtain maximal structural information.

MS/MS spectra were viewed using Thermo Finnigan software XCalibur (version 1.4), and loaded into Mass Frontier (version 4.0). An in-house, custom user library is built in Mass Frontier to store our reference standards' MS/MS spectra and their retention times. Once an unknown compound's MS/MS spectrum is obtained, its MS/MS spectrum is loaded into Mass Frontier and similar MS/MS spectra are searched against our reference standard library. If standard compounds are found whose match scores are greater than 900, then this unknown compound is considered to be the standard compound with the highest match score and the closest RT (i.e., RT difference between the unknown and the standard < 12 sec). Final identification was performed by purchasing candidate compounds and obtaining MS/MS spectra and comparing their spectra and RTs with those of the unknown compound and then finding the best match. In studies to date, the identities of about 60% of components that we have matched to reference standards have been confirmed by MS/MS. Of the remaining 40%, half were not detected in the ion trap instrument, as it has inherently less sensitivity than the TOF instruments.

Statistical analysis: Data were analyzed using Partek. T-tests were conducted on each component, comparing treated samples to controls for each dose group. Additionally, for 65 day whole blood samples, an ANOVA was conducted to identify components that were significantly perturbed by treatment. For visualization of the 65 day blood results, each subject in the treated group was first normalized to the average of the control group. Trends between dose and time points were assessed using two techniques. First, principal components analysis (PCA) was used to visually assess biological variability. Secondly, an unbiased quantitative assessment of the separation between the subjects in each dose-time group was conducted using an unsupervised learning approach based on hierarchical agglomerative clustering of the metabolomic profiles for the subjects. Pearson correlation and Ward's minimum variance method were used to determine cluster similarities. A probability value of < 0.05 was used as the critical level of significance for all statistical tests. To identify diagnostic or predictive biomarkers, globus pallidus manganese concentrations were categorized into 3 groups: values over 2 arbitrary units were considered High, values between 1 and 2 as Medium, and values less than 1 as Low. A Nearest Centroid classification was made using all 113 metabolites showing significant changes between dose groups in the ANOVA. The success of the classification was assessed using full leave-one-out, 1-level cross-validation and a normalized correct rate that averages the correct rate for positive and negative predicted samples.

Results

The LCMS method used in this study detected a total of 1097 parent peaks in whole blood and 2462 peaks in urine. Using the subset of 113 peaks that were found to be significantly changed according to the ANOVA analysis, principal component analysis (PCA) of the blood samples demonstrated good separation of the 3 dose groups at the 65 day timepoint (Fig. 1). The first 3 principal components captured a high proportion (70%) of the total variability. The major differences were between the high dose and lower dose groups, and were evident in the first principle component (PC1) which contained the largest proportion (35%) of the total variability. The two lower dose groups separated from each other on the PC2 and PC3, suggesting that the changes were more subtle.

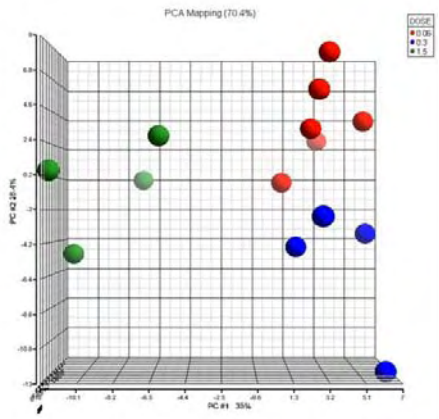

Figure 1: Principal component analysis of significant fold changes (vs. control) in metabolite profiles observed in whole blood from monkeys exposed to $MnSO_4$ at either 0.06, 0.3, or 1.5 mg Mn/m^3 for 6 hr/day, 5 days/week for 65 days.

The dendrogram and heat map produced by the hierarchical clustering analysis (Fig. 2) shows the log 2 normalized relative intensities of the individual components (peaks) for each subject. As in the PCA, subjects clustered perfectly according to dose group, and clear differences in intensities of groups of components are evident. These components, in turn, could be grouped into seven main clusters, as indicated by the dendrogram along the top of the diagram.

Table 1 shows statistically significant findings (fold changes and p values) from the t-tests comparing treatments to control in the metabolomic analysis of the 65 day monkey blood samples. The most consistent observations were in phenylpyruvate, which was increased in both the high and mid dose groups, and in allantoin and guanosine, which were increased in the mid and low dose groups. Changes in the high dose group at intermediate time points are shown in Table 2. Due to the absence of a matched control, these samples were compared to the 65 day air-exposed group. Any apparent changes may therefore be confused by an additional time variable, limiting the value of any conclusions. The results show a high degree of internal consistency, in that decreases in the vast majority of the components were observed at both 15 and 33 days. However, there was little consistency with the 65 day results, with the notable exceptions of decreases in aminobutyric acid and glutamine.

Observations from the urine samples are listed in Table 3. Conclusions from this data are limited because of the small number of observations in each group, but cystine appeared to be markedly depleted in both the mid and low dose groups. As above, urinary profiles at intermediate times were compared to the 65 day controls, and these results are shown in Table 4. As in the 65 day samples (Table 3), cystine appears to be markedly decreased.

Identities of the components shown in Tables 1 and 2 were confirmed by MS-MS fragmentation and the results are listed in Table 5. Of the 38 components that matched to a reference standard, 27 identities were validated as correct, 6 were incorrect and 3 were ambiguous. Two components did not provide sufficient signal in the ion trap MS for analysis. Only blood components whose identities could be confirmed are included in the tables.

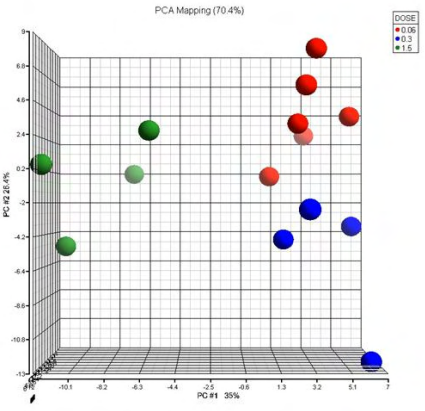

Figure 2. Hierarchical clustering of significant changes in metabolite profiles observed in urine from monkeys exposed to $MnSO_4$ at either 0.06, 0.3, or 1.5 mg Mn/m^3 for 6 hr/day, 5 days/week for 65 days. The scale to the right indicates log (fold change) relative to the vehicle control group.

Using the Nearest Centroid analysis, the subset of 113 significantly perturbed components predicted globus pallidus manganese concentrations with 72.9% accuracy for all treated and control subjects after 65 days exposure. Using only the five components that could be identified, the prediction rate was 70.9%. The individual profiles of these five components are shown in Fig. 3. In each case the high brain levels were predicted with 100% accuracy. On an individual basis, three of the identified were significantly correlated with manganese levels, according to Pearson's linear method. The correlation coefficient for guanosine was -0.81 (p=0.00008), for disaccharide (eg. lactose) was -0.69 (p=0.007), and for phenylpyruvate was 0.59 (p=0.026).

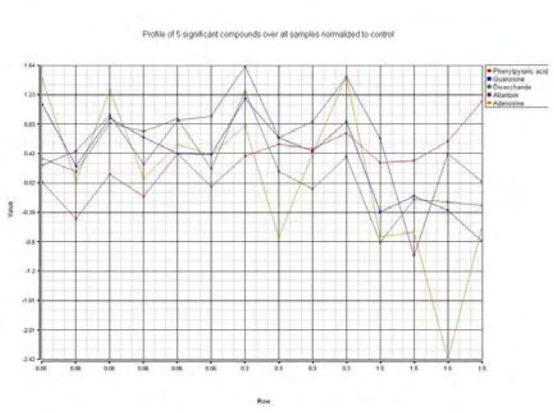

Figure 4. Profiles of identified biomarkers across all control and treated subjects. Values are normalized to the average of the control group.

Table 1. Significant changes (vs. air-exposed controls) in metabolite profiles observed in blood from monkeys exposed subchronically to $MnSO_4$ at 0.3, 1.5 mg Mn/m^3 for 6 hr/day, 5 days week. Blood samples were collected after 65 days of exposure.

Exposure group (mg Mn/m^3)	Treated/Control	p Value	Compound Name
1.5	1.56	0.0175	Phenylpyruvic acid
1.5	0.73	0.0231	Aminobutyric acid
0.3	4.21	0.0441	Indoleacetic acid
0.3	2.33	0.0019	Allantoin
0.3	1.86	0.0472	Hydroxybutyric acid
0.3	1.82	0.0248	Kynurenine
0.3	1.76	0.0055	Guanosine
0.3	1.48	0.0235	Uridine
0.3	1.46	0.0273	Phenylpyruvic acid
0.3	0.63	0.0123	Glutamine
0.06	1.59	0.0340	Allantoin
0.06	1.58	0.0098	Guanosine
0.06	1.49	0.0217	Disaccharide
0.06	0.62	0.0438	Proline

Table 2. Preliminary data showing significant changes (vs. air-exposed controls) in metabolite profiles observed in blood from monkeys exposed to $MnSO_4$ at 1.5 mg Mn/m^3 for 6 hr/day, 5 days week. Serum samples were collected after 15 or 33 days of exposure. As there were no control animals available at these time points, the treatments were compared to the 65 day controls above. Components (peaks) that were significantly altered at both 15 and 33 days are shown in bold.

Exposure group (mg Mn/m^3)	Exposure day	Treated/Control	p Value	Compound Name
1.5	33	0.85	0.0044	**Hexanoic Acid**
1.5	33	0.70	0.0010	**Nicotinamide**
1.5	33	0.67	0.0362	**Valine**
1.5	33	0.65	0.0361	**Glutamine**
1.5	33	0.64	0.0093	Alanine
1.5	33	0.63	0.0185	**Methionine**
1.5	33	0.59	0.0007	**Aminobutyric acid**
1.5	33	0.59	0.0231	Norepinephrine
1.5	33	0.59	0.0355	**Proline**
1.5	33	0.58	0.0008	**Hexose**
1.5	33	0.55	0.0131	Adenosyl-homocysteine
1.5	33	0.54	0.0143	Aspartic Acid
1.5	33	0.49	0.0018	**Arginine**
1.5	33	0.49	0.0001	**Glutamic Acid**
1.5	33	0.47	0.0081	**Acetylcarnitine**
1.5	33	0.47	0.0343	**Pyroglutamic acid**
1.5	33	0.45	0.0038	Oxidized glutathione
1.5	33	0.45	0.0026	**Pipecolic acid**
1.5	33	0.33	0.0002	**Deoxy GMP**
1.5	33	0.17	0.0003	**Hippuric acid**
1.5	33	0.04	0.0470	Creatine
1.5	15	0.89	0.0453	**Hexanoic Acid**
1.5	15	0.78	0.0380	Phenylalanine
1.5	15	0.74	0.0039	**Nicotinamide**
1.5	15	0.65	0.0035	**Aminobutyric acid**
1.5	15	0.63	0.0277	**Glutamine**
1.5	15	0.63	0.0393	**Methionine**
1.5	15	0.61	0.0329	**Valine**
1.5	15	0.60	0.0011	**Hexose**
1.5	15	0.59	0.0147	**Arginine**
1.5	15	0.58	0.0020	**Glutamic Acid**
1.5	15	0.56	0.0412	Citrulline
1.5	15	0.53	0.0134	**Pipecolic acid**
1.5	15	0.53	0.0303	**Proline**
1.5	15	0.52	0.0457	**Pyroglutamic acid**
1.5	15	0.47	0.0050	**Deoxy GMP**
1.5	15	0.42	0.0020	**Acetylcarnitine**
1.5	15	0.20	0.0010	**Hippuric acid**

Table 3. Preliminary data showing significant changes (vs. air-exposed controls) in metabolite profiles observed in urine from monkeys exposed subchronically to $MnSO_4$ at 0.06, 0.3 and 1.5 mg Mn/m^3 for 6 hr/day, 5 days week. Urine samples were collected after 65 days of exposure. Structural confirmation of this data set was not performed because of the limited number of urine samples available (1, 2 and 3 for the high, mid and low dose groups respectively).

Exposure group (mg Mn/m^3)	Treated/Control	p Value	Compound Name
1.5	1.49		Isochromanone
1.5	0.82		Acetylhistidine
1.5	0.49		Homoserine lactone
1.5	0.34		Hexitol
1.5	0.33		Pentose
1.5	0.30		Pentitol
1.5	0.29		Adipic acid
1.5	0.27		Mesaconic acid
1.5	0.26		Pentose
1.5	0.24		Hippuric acid
1.5	0.23		Kynurenic acid
1.5	0.23		Methyl glucopyranoside
1.5	0.23		Hydroxymethoxymandelic acid
1.5	0.21		Pyroglutamic Acid
1.5	0.18		Hydroxymethylpyrone
1.5	0.16		Pentose
1.5	0.15		N-Formylmethionine
1.5	0.15		Acetyltryptophan
1.5	0.15		N-Formylmethionine
1.5	0.14		Asparagine
1.5	0.12		2':3'-cyclic AMP
1.5	0.12		Uric Acid
1.5	0.10		Urocanic acid
0.3	0.62		Hexonic acid
0.3	0.57		Pentose
0.3	0.10		Cystine
0.3	0.34		Methionine
0.3	0.04		Hexosamine phosphate
0.06	0.79	0.0456	Isopentenyl Pyrophosphate
0.06	0.13	0.0003	Cystine
0.06	0.04	0.0036	Hexosamine phosphate

Table 4. Preliminary data showing significant changes (vs. air-exposed controls) in metabolite profiles observed in urine from monkeys exposed to $MnSO_4$ at 1.5 mg Mn/m^3 for 6 hr/day, 5 days week. Urine samples were collected after 15 or 33 days of exposure. As there were no control animals available at these time points, the treatments were compared to the 65 day controls above. Structural confirmation of this data set was not performed because of the absence of a matched control group.

Exposure day	Treated/Control	p Value	Compound Name
15	6.85	0.0003	Pentose
15	0.27	0.0455	Cystine
15	0.16	0.0246	Indolepyruvic acid
33	6.63	0.0452	Ketoglutaric acid
33	2.60	0.0398	Pyrocatechol
33	2.30	0.0338	Hydroxybutyric acid
33	1.95	0.0489	Hexose
33	1.91	0.0134	Hydroxyoctanoic acid
33	1.71	0.0029	Nicotinamide N-oxide
33	0.27	0.0371	Methylthiazoleethanol
33	0.23	0.0229	Cystine

Table 5. MS-MS confirmation of peaks with significant changes (vs. air-exposed controls) in metabolite profiles observed in serum from monkeys exposed to $MnSO_4$ at either 0.06, 0.3, or 1.5 mg Mn/m^3 for 6 hr/day, 5 days week. Serum samples were collected after 15, 33, or 65 days of exposure.

		MSMS Confirmation			
Peak Group	Initial Assignment	Correct	Probable	Incorrect	Unknown[1]
n_102.05_74	Aminobutyric acid	x			
n_103.04_107	Hydroxybutyric acid	x			
n_104.4_80	Serine			x	
n_114.06_96	Proline	x			
n_115.08_536	Hexanoic acid		x		
n_116.07_109	Valine			x	
n_121.03_393	Benzoic acid			x	
n_128.04_92	Pyroglutamic acid	x			
n_128.07_141	Pipecolic acid				x
n_130.06_98	Creatine	x			
n_132.03_74	Aspartic acid	x			
n_145.06_85	Glutamine	x			
n_151.04_538	Vanillin			x	
n_163.04_415	Phenylpyruvic acid	x			
n_164.07_339	Phenylalanine	x			
n_168.08_132	Norepinephrine	x			
n_173.1_92	Arginine	x			
n_174.06_476	Indoleacetic acid	x			
n_174.09_90	Citrulline	x			
n_178.05_368	Hippuric acid	x			
n_179.06_87	Hexose	x			
n_207.08_347	Kynurenine	x			
n_282.08_336	Guanosine	x			
n_308.1_92	N-Acetylneuraminic acid			x	
n_341.11_98	Disaccharide		x		
n_282.11_341	Adenosyl homocysteine	x			
n_611.14_92	Oxidized glutathione	x			
n_88.04_83	Alanine		x		
p_118.09_103	Valine	x[2]			
p_123.06_345	Nicotinamide				x

p_147.08_90	Glutamine	x			
p_148.06_78	Glutamic acid	x			
p_150.06_146	Methionine	x			
p_159.05_98	Allantoin	x			
p_204.12_128	Acetylcarnitine	x			
p_222.09_92	Acetylhexosamine			x	
p_245.08_314	Uridine	x			
p_348.07_312	Deoxy GMP	x			
Total	38	27	3	6	2

[1] Insufficient signal to obtain fragmentation pattern
[2] Mixture of valine, betaine and aminovaleric acid

Key Research Accomplishments

1. Changes in the serum metabolite profile seen in the $MnSO_4$-exposed monkeys identified several promising biomarkers related to oxidative stress and altered GABA and glutamate neurotransmission.
    a. The LCMS method used in this study detected a total of 1097 parent peaks in whole blood and 2462 peaks in urine
    b. Using the subset of 113 peaks that were found to be significantly changed according to the ANOVA analysis, principal component analysis of the blood samples demonstrated good separation of the 3 dose groups at the 65 day time point
    c. The most consistent observations were in phenylpyruvate, which was increased in both the high and mid dose groups, and in allantoin and guanosine, which were increased in the mid and low dose groups.
    d. Metabolomic analysis of serum from the $MnSO_4$-exposed monkeys revealed metabolite profiles indicative of oxidative stress.
    e. Identities of many of the components were confirmed by MS-MS fragmentation. Of the 38 components that matched to a reference standard, 27 identities were validated as correct, 6 were incorrect and 3 were ambiguous. Two components did not provide sufficient signal in the ion trap MS for analysis.
2. Conclusions from the urine data are more limited because of the small number of observations in each group, but cystine appeared to be markedly depleted in both the mid and low dose groups.
3. Confirmation of these potential biomarkers will require additional animal studies and/or epidemiological studies where other known biomarkers of exposure (e.g., brain MRI) have been examined.

Reportable Outcomes

- Manuscript:
    - Dorman DC, Struve MF, Norris A, and Higgins, AJ: Metabolomic analyses of body fluids after subchronic manganese inhalation in rhesus monkeys. *Toxicol Sci* (2008), submitted 2/08.
    - Struve MF, Turner KJ, Dodson PK, and Dorman DC (2007). Basal ganglia neurotransmitter concentrations in rhesus monkeys following subchronic manganese sulfate inhalation. Am J Indust Med **50,** 772-778.
    - Dorman, D.C., Struve, M.F., and Higgins, A.J., Identification of novel biomarkers of manganese inhalation. Society of Environmental Toxicology and Chemistry Europe 17th Annual Meeting, Porto, Portugal, May 24, 2007. [poster presentation].

Conclusions

As mentioned earlier, this study relied upon archived urine and serum samples collected at necropsy from monkeys subchronically exposed to $MnSO_4$. We previously reported an approximately 2.5-fold increase in the amount of manganese in the blood of monkeys exposed to $MnSO_4$ at ≥ 0.3 mg Mn/m^3 for 65 exposure days (Dorman et al., 2006a). Monkeys exposed to $MnSO_4$ at ≥ 0.3 mg Mn/m^3 for 65 exposure days also developed increased manganese concentrations in the olfactory tract, caudate, pituitary gland, kidney, lung, and pancreas (Dorman et al., 2006a). Increases in pallidal manganese concentrations were appreciated by brain MRI (Dorman et al., 2006b) and confirmed by atomic absorption spectrometry analysis of the tissues (Dorman et al., 2006b). MRI changes seen in our monkey study were similar to those reported in welders that have had

high manganese exposure and subsequently developed bilateral hyperintensity on T1-weighted images in the globus pallidus and other brain regions (Kim et al., 1999b; Sato et al., 2000). Monkeys exposed to $MnSO_4$ at the highest exposure concentration (1.5 mg Mn/m^3) for 65 exposure days also had increased manganese concentrations in the frontal cortex, trigeminal nerve, liver, skeletal muscle, and parietal bone. Importantly, exposure concentrations used in this study bracket the current threshold limit value for respirable manganese of 0.2 mg Mn/m^3 and are relevant to occupational exposures.

Metabolomic analysis of serum from the $MnSO_4$-exposed monkeys revealed metabolite profiles indicative of oxidative stress. Interestingly, manganese is incorporated into several antioxidant enzymes including manganese superoxide dismutase and glutamine synthetase. Moreover, it has been hypothesized that manganese-induced cytotoxicity may be due to oxidative stress; specifically, mitochondrial oxidative stress (for review see Taylor et al., 2006). Erikson and colleagues (2007) have recently completed assessments of biochemical endpoints indicative of oxidative stress in brain tissues from the monkeys used in the present study. These studies revealed that monkeys exposed to the highest exposure concentration had significantly lowered caudate glutathione (GSH) levels but significantly higher putamen GSH levels when compared to controls. The finding of increased levels of oxidized glutathione in the serum of these exposed monkeys is consistent with this central nervous system finding. Erikson and colleagues (2007) have also shown that glutamine synthetase protein levels in the cerebellum and frontal cortex were significantly decreased, but were increased in the putamen of monkeys exposed to 1.5 mg Mn/m^3.

Metabolomic analysis of serum from the $MnSO_4$-exposed monkeys also revealed metabolite profiles suggesting altered neurotransmission. In particular, metabolic profiling of the manganese-exposed monkeys revealed changes in γ-glutamyl and gamma aminobutyric acid (GABA) biochemistry. GABA is the most abundant inhibitory neurotransmitter in the adult brain (Beleboni et al., 2004). Glutamate is converted to GABA by decarboxylation via glutamate decarboxylase (GAD) and is degraded via GABA transaminase. In the present study, GABA and hydroxybutyric acid (a GABA degradation metabolite formed via succinic semialdehyde) had differential expression in serum from $MnSO_4$-exposed monkeys. Other neurochemical analyses have been performed on brain tissues from the cohort of animals used in the present study. Erikson and colleagues (2007) showed that subchronic exposure to $MnSO_4$ at ≥ 0.3 mg Mn/m^3 resulted in decreased caudate and cerebellum protein and mRNA levels of a glutamate transporter (GLT-1). Struve et al., (2007) found a marginally significant ($p < 0.1$) decrease in pallidal gamma aminobutyric acid (GABA) and 5-hydroxyindoleacetic acid concentration and caudate norepinephrine concentrations in monkeys exposed subchronically to $MnSO_4$ at 1.5 mg Mn/m^3 (vs. air-exposed controls). Findings in our nonhuman primates demonstrate some consistency with rodent experiments (for review see Fitsanakis et al., 2006). For example, Anderson and colleagues (2006) have shown that manganese accumulation leads to decreased GABA levels in the rat striatum apparently due to impaired uptake GABA uptake by striatal synaptosomes.

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